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Cytological Studies on Inflammation, Especially with Reference to the Origin of So-called Round Cells in Inflammatory Foci

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I. Introduction

The study on the origin of the mononuclear cells in inflammatory foci has made a great progress by the introduction of the technics of hematocytological examination, but on the other hand, the relation between the inflammatory cells and the blood cells seems to have come to be overestimated, leading to the advocation by some authors of theories asseverating that nearly all the cells appearing in inflammatory foci must be of hematocytic origin. These theories are indeed based on so precise cytological findings as not precedented in the past histopathological researches and have many followers among the modern researchers. In applying such theories, however, it is not to be denied that many a contradiction and inconsistency would be encountered in interpreting the various phenomena concerning inflammation. The largest cause of such foibles of these theories, I believe, lies in the neglect, slight or otherweise, of following further up the relation between the inflammatory cells and the local mesenchymal cells, especially the reticulo-endothelial cells (RE cells), and the metamorphosis of the latter cells—important points that should be discussed fully before entering upon theorising on the subject. In consideration of such negligence on these points, the present authors have undertaken a strict research, from both the angles of blood cells and tissue cells on the origin of mononuclear cells coming forth in inflammatory foci, in particular, typhoid cells in typhoid fever foci and epitheloid cells in tubercles, basing ourselves on the voluminous informations on the RES accumulated at this laboratory. As the result, we have succeeded in demonstrating that these cells, in refutation of the above mentioned theory,

are originated, not in blood cells, but in the RE cells, a summary of our study was reported at the " 7th Symposium on Hematology," 1954.[1),2),3)] Since then, we have proceeded with many fundamental studies on the transformabilities of the RE cells, and arrived at many informations that serve in endorsing our assumption, at the same time proving in concrete that the above theories are nothing but a system of speculation built upon a neglect of such transformabilities of the RE cells. The details of our study were reported by Prof. K. Akazaki in a special lecture at the general meeting of the Society of Dermatology and Veneorology of Japan in May 1956.[4),5)] The author will report hereunder in summary on the cytological informations on the metamorphosis of the RE cells obtained at this laboratory and make some discussions—in the various phases of the inflammatory process from the side of RE cells metamorphosis, as based on these informations.

II. State of Physiological Inflammation

1) Phagocytes in the serous cavities. It is well known that among the mesenchymal cells, in physiological conditions, the phagocytes in the peritoneal cavity are the nearest to the mononuclear cells observable in inflammatory foci in appearance, and that the some workers[6)] asserting the monocyte origin theory of these mononuclear cells have pounced upon this type of cells as the best example of wandering out into tissues and as the ground to build up their claim of monocytic origin of the mononuclear cells discovered in inflammatory foci. Murata's wide-scoped studies[7)] on these cells, however, has revealed that though these phagocytes in the peritoneal cavity look similar to blood monocytes they are quite different from the latter in their essential nature, and from the finding that these phagocytes are produced from the milky spots in the omentum majus formed in the embryonic stage already, they have been proved to represent local intraperitoneal phagocytes unrelated with blood monocytes. More recently, Koda has confirmed the constant existence of similar phagocytes in the thoracic cavity and the pericardial cavity and demonstrated that these are originated in the milky spots similar to those in the omentum majus and existing in the pleura and the epicardial wall. In consideration of their findings that these phagocytes are similar to the histiocytes in the subcutaneous connective tissue in their essential cytological characteristics but show also some quite dissimilar features (e.g., of being round and free, of showing nuclear lobulation and of forming neutral-red rosette), these authors hesitated to identify them with the subcutaneous histiocytes but tentatively set up a separate cell type for them under the name of the " phagocytes in the serous cavities."

2) Phagocytes in joint cavities. For elucidating the question whether

these phagocytes in the serous cavities are in fact of different type from the subcutaneous histiocytes prossessing characteristics sufficiently specific to distinguish them from the latter or these two apparently different types represent only two different phases of the same kind of cells in metamorphosis, we chose the joint cavities as the best field of study. According to the study reported by Yamagata[8] of Fukushima Medical College, 50–350 free cells per mm^3 are in permanent existence in the knee- and shoulder-joint cavities of normal rabbits and he found that about 70 per cent of these are of a type indentifiable with the phagocytes in the peritoneal cavity. Upon closer comparison of these joint cavity phagocytes with those in the abdomen, we found that while Murata's Type II cells (forming neutral-red rosette) are most prevalent in the abdominal cavity of rabbit, but his Type III cells (forming no neutral-red rosette) are more predominant in the joint cavities, where of the Type II cells only those showing no lobulation in their nuclei were conspicuous. Besides, spindle-shaped cells found only sparsely in the abdominal cavity were found relatively frequent in the joint cavities. Upon further studies directed to the synovial membrane, he found milky spots in permanent existense chiefly in its fat tissue tegumen, smaller indeed than those found in the omentum majus, and production of the joint cavity phagocytes from these milky spots. When 0.2 cc. of 10% peptone solution was introduced into a joint cavity, the cells forming such milky spots rapidly increased in number, accompanied by a corresponding increase of the phagocytes in the cavity and the number of Type II cells was found to rise therewith.

Since the joint cavities, viewed embryologically, are nothing but crevices in the connective tissues, different from the serous cavities, we are led to infer that the permanent existence of phagocytes of the same types in both these cavities means on the one hand that the phagocytes in the serous cavities are not cells of types specific to some specific locations and on the other hand that the phagocytes in the connective tissue, i.e., the histiocytes are transformable into the form of phagocytes in serous cavities. Furthermore, from the finding that the phagocytes in the joint cavities that may be looked upon as standing midways between subcutaneous histiocytes and serous cavity phagocytes come closer to resemble the serous cavity phagocytes morphologically by additional stimulation, the importance of the factors of such stimulation or activation in the process of metamorphosis of such connective tissue phagocytes is accentuated.

3) Vital staining. When rabbits are daily injected intravenously with 3.3 cc/kg of 1% trypan-blue solution for 10 days, the RE cells in the animals are stained nearly enough. By this method, we attempted to catch the process of transition of the subcutaneous histiocytes into phagocytes in the joint or the serous cavities by examination of all the RE cells

in the whole bodies of the animals. The results showed that the spherical histiocytes in the spleen, the bone-marrows and the lymph-nodes represented such transformed cells and besides that the histiocytes in the splanchnic capsules, the bulbar conjunctiva, the lamina propria of the intestinal wall and similar tissues were far more densely packed, more polymorphic and sometimes more frequently rounded out in form than those in the subcutaneous connective tissue. Such a polymorphism of the histiocytes in many different regions is sufficient to justify the inference that the gaps between the forms shown by the histiocytes in the subcutaneous connective tissue, the joints and the serous cavities cited above are filled up hereby and that the different forms of these cells are only different phases of the same type of cells in transformation.

4) *Physiological stimulation.* Not to speak of the spleen and the lymph nodes, it is easy to suppose that such tissues as the bulbar conjunctiva and the propria of the intestinal wall are normally under stronger stimulations than are the subcutaneous tissues and we need not mention that the joints and the serous cavities are all constantly in the state of mechanical stimulation. It is probable that such incessant stimulations exert some decisive effects on the forms of the histiocytes in the parts concerned. For obtaining informations on this subject we conducted the following experiments:—*a*) When 1 cc India ink was introduced into the abdomen of mice, carbon particles were found in the thoracic cavity and partly taken in by phagocytes there in 10 min.. *b*) When 0.2 cc of India ink was directly introduced into their thorax, in 30 min. lumps of carbon particles were found adhering mainly on the surface of the pleura over the mediastinal cavity and some of the particles penetrated deeper into the membrane and began to be taken in by phagocytes. *c*) When mice were made to inhale soot for 3 hours, a proliferation of the milky spots in the pleura began soon after the treatment and in 18–48 hours accumulation of carbon particles was observed, first in the intrathoracic free phagocytes and then in the milky spots in the pleura lining the mediastinal cavity. When 0.1 cc of India ink was introduced into the trachea of mice, similar findings were obtained in 1–48 hours. These results seem to suggest that the pleural cavity exchanges humor with the abdominal cavity and the lung, mainly through the route of the mediastinal cavity, and the intracavital free cells and the cells forming the milky spots in the pleura are constantly active in disposing of such incoming humor. Thus, the phagocytes in the thorax are under incessant stimulation and may be looked upon to remain always in a state of hyperfunction. Similar states of excitation may be easily guessed to exist in the abdominal, the pericardial and the articular cavities too (Experiments on this subject planned).

Accordingly, we may say that all the different regions and tissues in

the body are constantly under stimulation, strong or weak, the RE cells are drawn into reactions and are activated in accordance with the kind and intensity of the stimulation to which their respective sites are subjected and they take up the different cellular forms as the concrete expression of their state of heightened function.

Now, if regard inflammation from the site of cellular response and take it to be a state where an abnormally strong mobilization of the inherent function of the cells is demanded, we may regard the serous and the joint cavities described above to be in a state of inflammation physiologically. This state we understand to fall under the appellation of physiological inflammation as proposed by Roessle[9] and emphasised by Maximow.[10] Therefore, we can easily suppose that even the subcutaneous connective tissue cells, when an abnormally high functioning is demanded, would take the form similar to or identical with the histiocytes in these cavities. Accordingly, the fact that there is a resemblance between the mononuclear cells found in inflammatory foci and the phagocytes in the serous cavities does not result from the appearance of a specific type of cells called the intra-abdominal phagocytes in such foci, as many authors have opined in the past, but indicates that both the inflammation cells and the intra-abdominal phagocytes represent morphological expressions of one and the same cellular type, that is, of the histiocytes equably in a state of hyperfunction.

III. States of Stimulation

When the RE cells are abnormally activated and proliferate so that the general condition of the site shows a deviation from the normal scope, we look upon such a condition as a state under stimulation. This state may occur in all the RE system in the body or may be limited to some specific organ or tissue. The former state we wish to call the general and the latter the local state of stimulation. In this meaning, the so-called inflammatory foci may be said to represent, from the view-point of the RES, a quantitatively very intense but locally circumscribed state under stimulation.

The state of general stimulation.[11] The subcutaneous tissue is normally subjects to extremely weak stimulations only, so that the histiocytes here may be said to be in the form expressing a state of rest. But when we began examining the subcutaneous tissues of a number of untreated adult rabbits and mice, we found that the form and the number of the histocytes therein were rather widely different by individuals and in some they were in a state of very high activation. By systematic examination, we found that even among the untreated normal animals the level of activation and cell proliferation in the entire RES was different

individually and the subcutaneous histiocytes were unequally activated and proliferated according to the different level of RES excitation, that is, we found some among the untreated animals were already in a state of general stimulation.

Searching for a method of experimentally inducing such a state of abnormal activation of the RES in general, we found that a single intra-abdominal, inoculation of 5 cc/kg of typhoid vaccine was the simplest and surest method with rabbits. Following such a treatment, the histiocytes were found to undergo manifold transformation and proliferations. Since no emigration of leucocytes occur throughout, we could follow up the transformation processes of the histiocytes without being troubled by the interference of leucocytes. The degree of transformation of the subcutaneous histiocytes following inoculation of typhoid vaccine is markedly affected by the original level of RES activation of the animal, but in a rabbit apparently in relative normal health, the following progress seems to be the usual course. After the inoculation, the nuclei and the cell bodies of the histiocytes in existence begin to diminish in size and the focal arrangement of these cells becomes conspicuous. Soon, the condensed histiocytes showing such an arrangement begin to separate into solitary free cells and become gradually spherical, while many vacuoles appear in the cell bodies, which soon disappear and on the 3rd or 4th day a picture of degeneration comes to the foreground. A perceptible proliferation of histiocytes is usually observed on the 5th or 6th day. Before the reappearance of such histiocytes, large-sized cells of variable but predominantly spindle-like form comes into conspicuous appearance which increase gradually in number by active mitosis. These cells have large round or oval nuclei and their bodies are ample, often being linked together as if in mutual syncytium. The mitochondria of these cells are of short thread- or rod-form and are scattered throughout the cell bodies, but gradually take on short rod-like or granular form and gather densely around the nuclei and when minute neutral-red granules come to increase in number and mix with the former granules, the extracellular part of cytoplasma falls into vacuolar degeneration, soon the cell body is cut off from the vacuolated part and form a single cell and then is converted into young fixed histiocytes by subsequent contraction. The young fixed histiocytes thus formed are then proliferated by division (chiefly amitosis), resulting in focal or fascicular formation of proliferated histiocytes. We gave the name of prehistiocytes to such cells representing the preliminary stage of histiocytes. Those cells almost totally lack in phagocytosis, are scarce and hard to define in normal subcutaneous tissue of rabbit, but increase enormously in the state of stimulation and become the main factor of histiocytic proliferation. The multifariousness in form of the cells coming

forth in the course of such proliferation and the manifold combination of the transformation processes of these cells into histiocytes calls the multiform hitiocytes into existence. The small-sized histiocytes appearing under stimulation represent one of the types of such multifarious new-born histiocytes and comprise many kinds including those directly transformed from prehistiocytes on the one end and those formed by division of histiocytes on the other, showing diverse forms according to their genesis. In the former type, the cells are rich in mitochondria, are predominantly free and are often negative in vital staining. The latter have bodies filled with neutral-red granules, are closely similar to the stable large-size histiocytes in appearance and show active phagocytosis. From these observations, we may summarize our cytological findings on the subcutaneous tissue under stimulation into the following major items:—*a*) Increase of the cells in number. *b*) Proliferation of prehistiocytes. *c*) Growth of histiocytic nidal or fascicular proliferation foci. *d*) Generation of numerous small-sized round free histiocytes. From the observed fact that the occurrance and the intensity of these major findings show approximate parallelism to the level of activation and proliferation in the entire RES, we may say these findings offer concrete means for estimating the general state of

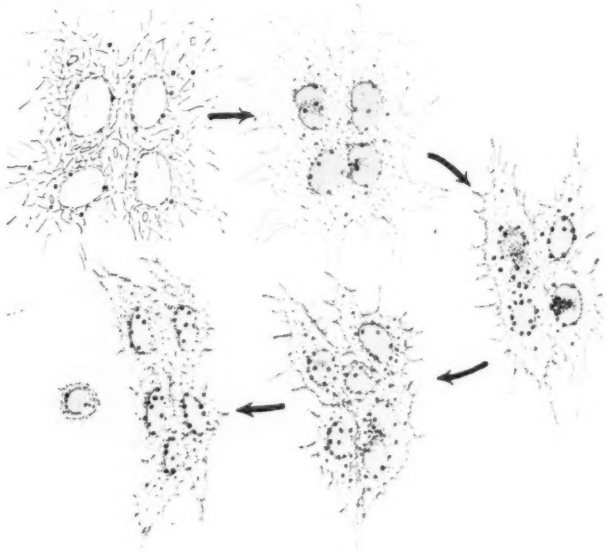


Fig. 1. Schematic representations of inversion of prehistiocytes into spindle-shaped and rounded histiocytes. Supravital staining with neutral-red and Janus-green.

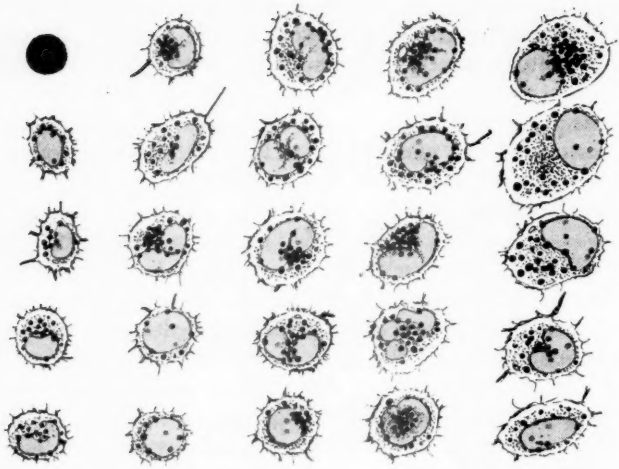


Fig. 2. Free rounded histiocytes from the subcutaneous connecture tissue of a rabbit injected intraperitoneally with typhoid vaccine. Supravital staining with neutral-red and Janus-green. The black corpuscle at upper left side shows a red cell.

RES activation.

IV. Vicissitudes of the Histocytes in Inflammation Foci (Localized Stimmulated Condition)

The phlogogenic agents used in our experiments consisted in the animal's own blood, 1% cholesterin added to a Tween 80-physiologic saline solution, 1% agar solution, silica dust, bone powder, typhoid vaccine, horse serum, BCG suspension, lepromin etc.. These agents call forth more of less characteristic responces respectively, but the inflammatory reactions caused by introduction of these substances have the common feature of being free of undue interference of leucocytes, so that the transformation process in the histiocytic cell reaction can be clearly grasped. We will refrain here from entering upon the details of the individual inflammation processes, and describe only the fundamental type of the transformation of the histiocytes coming into existence during the process.

The first change to be observed is the initial reaction coming forth just after the introduction of the inflammatory agent down to the occurrence of leucocyte infiltration. Immediately after the introduction, the subcutaneous tissue begins to swell and becomes edematous, while most of the existing histiocytes are reduced in size. The cell nuclei are pycnotized, becoming round or of elongated-ellipsoid form. The cell bodies are also diminished in size, become spherical, spheroidal or of

long spindle-form and filled with coarse neutral-red granules, while numerous unstainable vacuoles also make appearance. Soon the round free cells begin to increase, the cord-wise arrangement at first prominent becomes cut up and the cells become solitary and free. Meanwhile the fibrocytes also mostly fall into degeneration and come to be replaced by large-sized, slightly more basophil cells with elliptical nuclei rich in chromatin. Leucocyte infiltration comes in usually at this stage, that is, 1–3 hours after the introduction of the inflammatory agent. The infiltration of granulocytes reaches the maximum intensity in 6–12 hours, then falls off gradually, while the picture of degeneration becomes frankly distinct, and the leucocyte infiltration fades out from the inflammation focus on the 2nd or 3rd day. The emergence of blood monocytes was usually slight in the inflammation foci induced by the above-mentioned agents. These monocytes come out together with the granulocytes and mixed with them in the inflammation foci, and take in the latter in phagocytosis but disappear from the foci following their extinction. The histiocytes in such foci of leucocyte infiltration have their bodies swollen up and mostly die away, but in the sites at some distance from the center of leucocyte infiltration, the cell proliferation process similar to that in the state of stimulation cited above in form but far more vehement in degree is rapidly developed. A prominent focal proliferation begins, releasing round or spheroidal cells, mainly consisting in monocytoid cells, while the chromatin in the larger-sized fusiform cells are increased, both those size and form become more various and those cell bodies become more basophil. These large-sized cells are incessantly changed over into medium- or small-sized cells while they proliferate rapidly themselves, so that the focal cells come to comprise more and more younger cells. The invasion of such cells into the central part of the foci depends on the level of devastation of the foci. In the cases of small and mild foci, highly basophil cells of mainly spindle-form penetrate into the necrobiotic foci while undergoing rapid proliferation and utilizing the remnant collagenous fibres and fibrine masses as footholds. The nuclei of these cells are rich in chromatin, frequently contain nucleoli and many figures of nuclear division are also observed. The round cells of varying sizes born in the course of cell proliferation infiltrate into the liquid past devoid of trabecular tissues. These free cells in rare cases are observed to undergo mitosis, but in most cases the new cells seem to proliferate further through amitosis, and in conformity with this observation we found many nuclei in indefinite lobulation. The foci mainly containing large-sized fusiform cells mixed with large and small round cells then make transition into foci mainly filled with small-sized round cells. In the case when the former stage persists for 3–4 days, this transition occurs on the 4th–6th day. These free cells are similar to lym-

phocytes, monocytes or plasmocytes in appearance, but are dissimilar from any of them in that they show active phagocytosis. As the liquid content in the focus is decreased, the number of the small-sized round cells is also reduced, while the medium-sized formed under the same mechanism as the former become predominant. The bodies of such cells gradually lose their characteristics of free cells, the existence of matters taken in by phagocytosis become rarer to observe and the circumscription of the focus becomes more marked. The circumscribed focus remains a while in the stage of predominance of medium- and small-sized cells, but in other sites the free cells become very rare and the nuclei of the large-sized spindle-form basophil cells hitherto in predominance become uniformly round and medium-sized and lose in their chromatin contents. Their cell bodies become multipolar and more acidophil, and their mutual boundaries become blurred. These-after, the degeneration of these cells becomes more prominent, their number falls off rapidly and slowly the normal tissue is restored. In the interim, the findings observed in the subcutaneous tissues under general stimulation above is retained over a long period. The remnant localized foci sometimes show the structure resembling milky spots, wherein also the predominance of small-sized round cells is gradually usurped by multiform cells, the picture of degeneration becomes more prominent and belatedly the normal state is reinstated here too. The above-cited free round cells are modified into different forms by application of different agents. E.g., when cholesterine solution was administered, vesicular cells were found in predominance, after BCG suspension, lymphocytic cells of varying size were most frequent and after Mitsuda's reagent solution for leprosy, plasmocytoid cells were most numerous in the acme stage.

The above-described vicissitudes in the RES response in inflammatory foci may be schematically represented as in Fig. 4. In the uppermost line is indicated the intensity of the stimulation, where N means normalcy and R the stimulation. The phagocytic histiocytes are shown above and the prehistiocytes below the horizontal medianline. The existence of prehistiocytes is not clear in normal state, fixed-type histiocytes being predominant and nearly no free round cells being observable. As the intensity of the stimulation increases, the prehistiocytes come into more pronounced existence and they become more active. At the same time, histiocytes are actively produced, of which the free round-type cells gradually rise to majority in number. When the stage of maximum stimulation (R##) is attained, the most activated and young prehistiocytes become highly predominant and the free cells also rise to the maximum in number (In actuality, the maximum predominance of the free round cells comes a little later than that of the prehistiocytes). With the abating of the in-

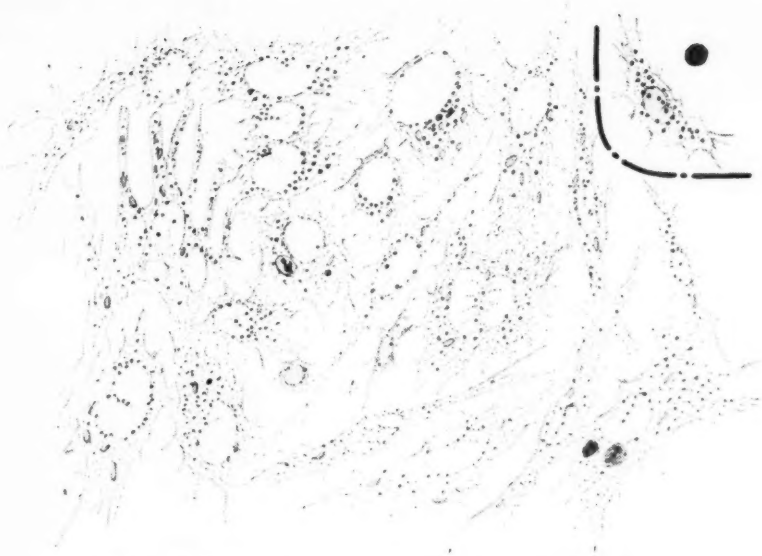


Fig. 3. Prehistiocytes from the inflammatory foci caused in the subcutaneous tissue of a rabbit. New-born histiocyte in upper right side. Supravital staining with neutral-red and Janus-green.

flammatory stimulation, the activation and rejuvenation of the prehistiocytes also gradually fall off, their number decreases, and the number of free round cells also decrease in parallel, the fixed-type cells are augmented and the normal state is approached. Then these fixed-type cells too are reduced in number, while natured cells gradually take the load and finally the normalcy is restored. The above summary has sketched the temporal variation of the metamorphoses in RES accompanying the progress of inflammation, but the same schema is applicable in studying differences in the cytological findings in different positions of the inflammatory sites subjected to a different degree of RES stimulation. So, let the condition in the stage of R## stand for that in the focal center and N± for the sites at a distance too large for the influence of the inflammatory stimulation to reach, the state of cellular reaction in the sites, near to and far from the focal center between there two positions can be read in the schema. Of cousc the breadth and the rise and fall of the curve should be different with different intensity of the stimulation applied. Besides, we must point out that the data indicated in this schema are applicable to the cases of physiological inflammation as well. Namely, we can adapt the state under N± to that in the normal subcutaneous tissue and then the forms

of histiocytes given under R### to those in the abdominal and the pleural cavities and in the spleen, those under R## to the phagocytes in the joint cavities and identify the state either of R+ or R## with that in the propria of the intestinal mucosa, the conjunctiva or the splanchnic capsules. In the light of such observations, we can readily understand the morphological similitude of the round cells in the inflammation foci and the phagocytes in the serous cavities cited above.

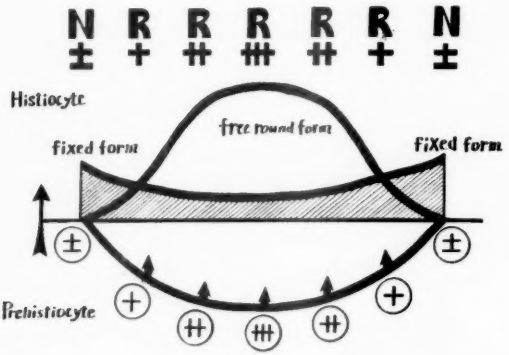


Fig. 4. Variation of the formation of histiocytes in inflammatory foci.

V. Relation between Histiocytes and Other Cell Types

As described above, the histiocytes go through rather kaleidoscopic changes in inflammatory foci following the progress of inflammation, so the discrimination between the histiocytes and cells of other origins naturally becomes an important operation.

1) Histiocytes versus monocytes. We have reported on the vicissitudes of monocytes in inflammatory foci on various occasions, and in our present experiments, we could confirm that the monocytes coming forth subsequent to the infiltration of granulocytes are capable of considerable phagocytosis, and at the same time we could reaffirm our original contention that these monocytes do not further divide and proliferate but always eventually degenerate and disappear. On the other hand, the monocytoid transformation of the histiocytes runs different courses in different stages. First, the small lobulated cells formed prior to the emergence of the granulocytes and the cells of the same type released from the nidal proliferation of surrounding tissue at the stage of appearance of granulocytes have their bodies filled with granulocytic granules and thus show an appearance dangerously similar to the monocytes filled with similar granules. The new-born prehistiocytes produced in the vortex of granulocyte

Fig. 5

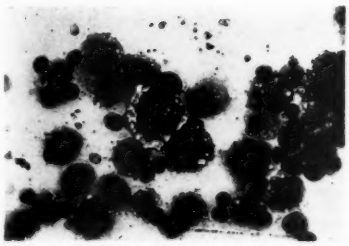


Fig. 7

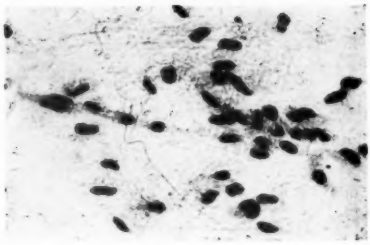
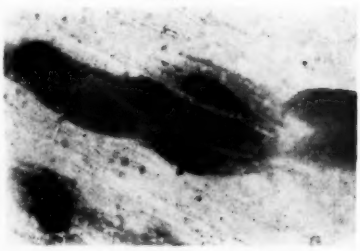


Fig. 6



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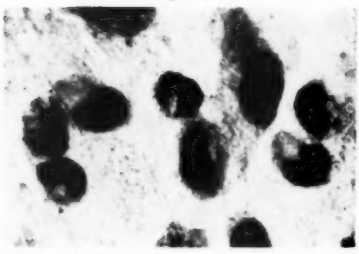

Fig. 5. Remarkable carbon-containing macrophages from pleural cavity of a mouse 5 days after inhalation of lamp-soot for 3 hours. Giemsa stain.

Fig. 6. Strech preparation of the subcutaneous tissue of the non-treated rabbit. Focal arrangement of histiocytes. Giemsa stain.

Fig. 7. Strech preparation of the subcutaneous tissue of a rabbit 24 hours after injection of 10 cc of typhoid vaccine intraperitoneously. Focal and cordal arrangement of histiocytes and appearance of new-born histiocytes and prehistiocytes in the focal arrangement. Giemsa stain.

Fig. 8. New-born histiocytes having kidney-shaped nucleus similar to monocytes. From subcutaneous tissue of a rabbit 2 days after local injection of Mitsuda's lepromin reagent, which was previously injected with promin intravenously. Giemsa stain.

infiltration display active phagocytosis without delay. The nuclei of such cells preferentially show various kinds of lobulation successively. Their chromatin nets are delicate and fine, their bodies contain much matter taken in phagocytotically and always show more or less advanced degeneration. These also look closely resembling monocytes under Giemsa's staining, though larger in size. The cells of the two types above are observed in large number in the stage of declining granulocytes in ordinary inflammation cases, and the coincidense of this stage with that of the acme of the monocyte level curve may be especially noteworthy. We have succeeded, as reported below, in expediting or retarding the onset of such a stage of monocytoid cells reaction by previously activating or depressing the function of the RE cells, and such results also convinced us of the

Fig. 9

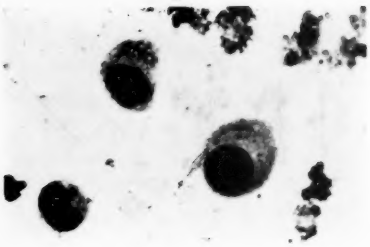

Fig. 11

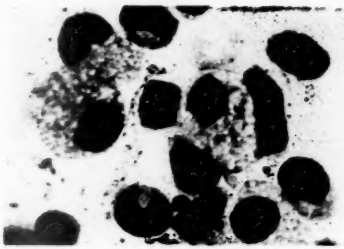

Fig. 10

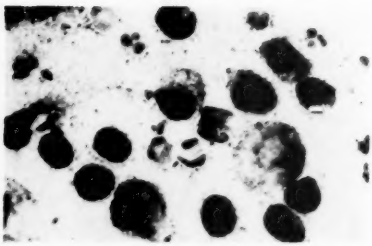

Fig. 12

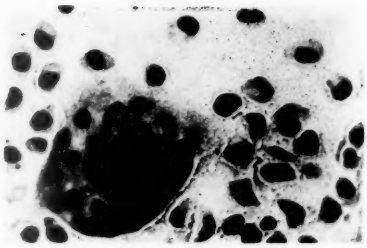

Fig. 9. Appearance of two histiocytes similar to plasma cells and a monocyte-like histiocyte. From subcutaneous tissue of a rabbit 5 days after local injection of 1% aquous solution of agar. Giemsa stain.

Fig. 10. Accumulation of new-born histiocytes similar to lymphocytes. From the cover glass 2 days after inserted into the subcutaneous tissue of a rabbit, which has been previously injected with cardiolipin-lecithin intravenously. Giemsa stain.

Fig. 11. Foam cells showing transitional varieties among surrounding hystiocytes similar to monocytes or lymphocytes. From the cover glass 2 days after inserted into the subcutaneous tissue of a rabbit, which was previously injected with cholesterin intraperitoneally. Giemsa stain.

Fig. 12. Giant cell formation on a cover glass after 4 days inserted into the subcutaneous tissue of a rabbit, which was previously injected with promin intravenously. Giemsa stain.

histiocytic origin of these cell types. The monocytoid cells released from the above prehistiocytes in the stage of further activation where they have turned into cells with gigantic and coarse nuclei and strongly basophil bodies, are usually small-sized cellules with pycnotized bilobular eccentric-standing nuclei, rich in mitochondria and frequently show neutral-red rosette formation. These cells usually come forth prior to the lymphocytoid or plasmocytoid histiocytes described below and their lobulated nuclei seem to suggest the amitosis. The appearance of their cytoplasma supravital staining coincided with that of lymphocyte-type or plasmocytoid histiocytes. They show generally feeble phagocytic ability.

In the above, we have described in summary three types of monocytoidized histiocytes, their different types are due to the difference in the characteristics of the prehistiocytes in different stages. These cells sometimes show the appearance of being different in kind from the fixed histiocyte in the vicinity, due to secondary migration and aggregation, but a nearer study of the specimens will give multiple evidence of the release and production of these from the prehistiocytes.

2) *Histiocytes versus lymphocytes and plasmocyte.* The rounded histiocytes showing some or all the features of eccentric-standing nuclei rich in chromatin, highly basophil cell bodies and clear field around the nuclei would look closely resembling plasmocytes, but the conspicuous phagocytic ability denies the possibility of these type of histiocytes changing over into plasmocytes proper. Besides, the more or less variable size of these cells, the gray tint seen under May-Giemsa's staining in the basophil bodies, the prominent nuclear membrane, the fine nuclear nets, the frequent transition of the nuclear form to the oval, the rosette formation by minute neutral-red granules under supravital staining, the enormous number of mitochondria found in the ample cell bodies, of these cells are findings essentially dissimilar from the findings obtained from the plasma cells discovered simultaneously in many sites of the same body. Secondary transformation of such plasmocytoid histiocytes (vesicular cells, epitheloid cells etc.) seem to characterize some kinds of foci.

Similarly, the lymphocytoid cells are identical in form with the phagocytes Type I in the joint cavities, can pass over into either plasmocytoid or monocytoid histiocytes and it is difficult to discriminate between these three types of cells. Cells of these three types probable constitute the essential elements consisting the round-cell infiltration or the so-called lymphocytic infiltration in inflammation foci.

3) *Histiocytes versus giant cells.* We have already elucidated in our studies on tuberculous foci and experimental subcutaneous inflammation induced by introduction of bone powder[12] that the multinuclear giant cells observed in inflammation foci are derived from the RE cells and pointed out the errors bidden in the results of Forkner and Ikuta's studies, hitherto cited as the mainstay of the monocytic origin theory of these cells, so that we are convinced a finis has been placed on the problem of the genesis of these cells. In the present study, we have mainly proceeded with researches on the histogenetic development of giant cells. The results in summary showed that the formation of giant cells may be interpreted in a row with the focal formation of histiocytes in these proliferation process and may be attributed to a miscarriage in the course of their proliferation process. Under stimulation, we have found a few syncytic cells consisting mainly of prehistiocytes, but the stage of highest production of giant cells

is the stage where the highly basophil spindle-form cells are actively proliferating and at the same time releasing round cells of varied size in full activity. In this stage, when the active proliferation of cell nuclei overreaches the speed of cell division, the giant cells can be produced. Consequently, the nuclei of the giant cells thus produced are identical in nature with the nuclei in the round mononuclear cells existing in the vicinity, their cell bodies are also highly basophil, often contain similar neutral-red rosettes, though larger in size and similarly contain very abundant mitochondria.

Even the histiocytes once turned round and free may in some cases become polynuclear cells by nuclear division, but in such cases, the number of such nuclei is limited below 5 or 6 and their cell membran is more definite than in the cases of the above giant cells. The new-born giant cells go on increasing in the number of nuclei for a short time after their production, but soon their nuclei become swollen, the amount of chromatin decrease, unstainable vacuoles begin to appear in their cytoplasm and their mitochondrias diminish in quantity paralleling the reduction of their basophilie and the alterative findings become conspicuous, then before long these giant cells also finally disappear from the inflammation focus as do all the other free round cells. The phagocytic capacity of these giant cells shows a parallelism with the youngness or maturity at the time of their formation and in the time thereafter.

VI. Conclusion

1) The RE cells in the living organism are more or less activated and show a variety of metamorphosis according to the functional demands made upon them in their respective sites. Thus, the morphological dissimilarity between the histiocytes in the serous cavities and the subcutaneous tissues represents only a difference in the phases of such metamorphosis, the form of the former being due only to the higher stimulation to which the serous cavities are constantly subjected to. Consequently, when the histiocytes in the subcutaneous connective tissue are under stimulation of a comparable intensity, these will also take upon a form resembling those in the serous cavities. Accordingly, we believe it is a mistake to presume that the similarity of the mononuclear cells observed in the inflammation foci to the phagocytes in the serous cavities leads to the interpretation that some phagocytes from the serous cavities—a specific cell type— or blood monocytes have come into the foci, as many have opined in the past, but in reality these represent nothing but a specific form of cells of one land the same type under stimulation. The state where the histiocytes show an inflammatory form in normal conditions too, as do the phagocytes in

the serous cavities, we understand to fall under the idea of " physiological inflammation " as proposed by Rössle and Maximow.

2) The RE cells are thus activated to extents variable with their respective locations, but there is of course a natural limit of normalcy to such an activation. The state when such a normal limit is surpassed we have called the state under stimulation. Having induced a state under stimulation experimentally and studied the response of the entire RES in such a state, we found that we can approximately estimate the level to which the entire RES is activated by examining the metamorphosis picture of the subcutaneous histiocytes. At the same time, through cytological examination of the metamorphosis of the subcutaneous histiocytes in such a state, we succeeded in ascertaining the fundamental processes of the multiple metamorphosis, such as the transformation of the subcutaneous histiocytes into round and free cellules, occurring in the inflammation foci, without being troubled by the interference of blood cells. During the experiments, we noted the existence of prehistiocytes serving as the sources whence the histiocytes are proliferated. In summary of our outstanding findings concerning the subcutaneous tissues under stimulation, we can point out the increase of prehistiocytes in number, the focal proliferation of the histiocytes seemingly due to the local proliferation and metamorphosis of such prehistiocytes and the increase of small-sized round histiocytes derived from the pre-existing histiocytes or directly from prehistiocytes.

3) From such a view-point, we may interpret the inflammation induced in the subcutaneous tissues by introduction of phlogogenic agents to represent a state of localized but highly intense stimulation. In such a case, the apparent course of transformation of the local histiocytes is essentially indentical with that in the state of general stimulation described above, but far more intense in degree. Here, the activation and proliferation process of the prehistiocytes becomes much stronger, both quantitatively and qualitatively, their transformation into histiocytes goes on much more actively and quickly, and the histiocytes produced consist mainly in round free cells of varying size, fixed-type cells being sparse among them. With the abatement of the stimulation, however, the level of prehistiocyte activation also falls off and the conversion into fixed-type histiocytes comes gradually into the fore-ground.

4) The round and freed histiocytes outwardly resemble the monotyes, the lymphocytes and the plasmocytes rather closely, but on nearer examination, are found to be not identical with any of these cells, so that we are not inclined to support the theories postulating the transition of the RE cells into such cell types. The formation of giant cells is attributable to a miscarriage in the course of focal proliferation of such histiocytes, and the chance of such misformation is large in the stage when the pre-

histiocytes are in active proliferation and also when the small-sized round cellules are rapidly produced. The giant cells thus produced follow the same fate as the free round cells and disappear before long.

References

1) Hasegawa, H., Acta haem. Jap., 1954, **17**, 433.
2) Uekane, K., *ibid.*, 1955, **18**, 107.
3) Akazaki, K. & Kojima, M., 1954, 7th Symposium on hematology, 121.
4) Akazaki, K., Hiseishi (Jap.), 1956, **66**, 376.
5) Akazaki, K. & Kojima, M., Nihon-Rinsho (Jap.), 1957, **15**, 5.
6) Amano, S., Ketsuekigaku-no-kiso (Jap.), Vol. 1, Tokyo, 1949.
7) Murata, J., Acta haem. Jap., 1955, **18**, 38.
8) Yamagata, Y., Tr. Soc. Path. Jap., 1956, **45**, 297.
9) Rössle, R., Verh. dtsch. path. Ges., 1923, **19**, 18.
10) Maximow, A., Arch. Path., 1927, **4**, 557.
11) Kojima, M. & Watanuki, T., Tr. Soc. Path. Jap., 1956, **45**, 389.
12) Ogata, M., *ibid.*, 1956, **45**, 390.

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Effect of Denervation of the Cat's Sweat Glands on Their Responsiveness to Adrenaline, Nicotine and Mecholyl

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It is known that many structures are rendered by deprivation of their nerve supply supersensitive to the transmitter substances of the nerve impulse and to other chemical agents. This was formulated by Cannon[3] in 1939 as a law of denervation. However, the sweat gland, at least in man, appears to be one of apparent exceptions to this law.

With regard to the effect of denervation on the excitability of the cat's sweat glands, information is still incomplete. In 1877 Luchsinger[17] first described that the response of the sweat glands of the cat's foot-pads to pilocarpine diminished progressively after denervation of the glands by section of the peripheral nerve. This observation was confirmed by Nawrocki,[18] Vulpian,[27] Ott[19] and Langley[15] On the other hand, Burn[1,2] and Simeone, Mentha and Rodrigues[25] observed not infrequently that the cat's sweat glands became supersensitive to pilocarpine during a certain period after denervation.

The present study deals with the effect of denervation of the cat's sweat glands on their responsiveness to adrenaline, nicotine and mecholyl (acetyl-beta-methylcholine).

Experimental

Methods

Observations were made on unanaesthetized cats with unpigmented hairless pads of the hind feet. Forty-nine cats of both sexes were used. The body weight of the animals ranged from 1.1 to 4.0 kg, except one weighing 0.3 kg. Before using them, it was ascertained that large number of the sweat glands of the pads of these animals retained well the ability to sweat spontaneously. In order to exclude sweating of central origin, a segment of the sciatic nerve about 2 to 3 cm in length was excised at the level of the great trochanter under ether anaesthesia. In the majority of the cats used, this operation was performed asepti-

cally on both sides at one or two sittings.

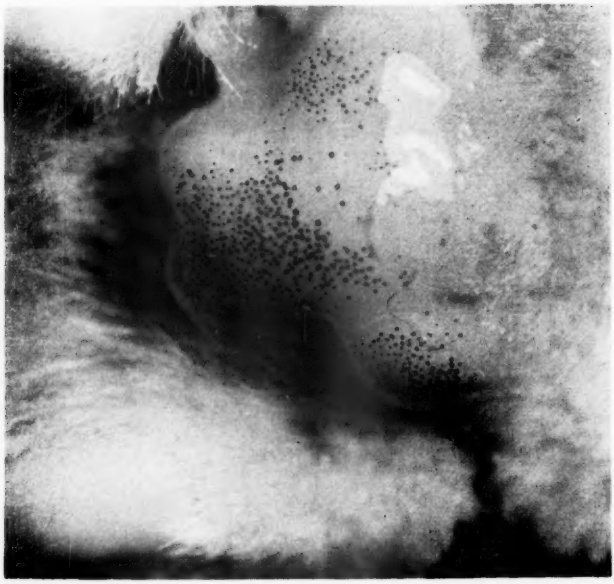

Fig. 1. Local sweat response to adrenaline in the cat's foot-pad. Subcutaneous injection of 0.1 ml of 10^{-4} adrenaline was made into the central pad from the front on the first day of the sciatic nerve section. Sweating was visualized as black spots by the method of Wada and Takagaki. Magnification: ×5. Unretouched.

Sweating was visualized as black sweat spots at the pores of active sweat glands by the iodine-starch method of Wada and Takagaki,[32] as illustrated in Fig. 1. The pads to be tested were painted first with a 3% iodine-absolute alcohol solution. Then, the area was coated with a 50% emulsion of corn starch in castor oil. The sweat response produced by test agents was observed with a magnifying lens (×16). When required, the duration of the sweat response was determined by repetition of wiping the sweat spots with cotton pledgets and subsequently painting the test area with the reagents for visualizing sweating, at intervals of 5–10 minutes after injection. The duration was approximately expressed by the interval between the time of the appearance of the response and that of the final application of the reagents which was made before the disappearance of the response.

In a first series of experiments the responsiveness of the sweat glands to local injections of adrenaline, nicotine and mecholyl was studied by measuring minimum effective concentrations of these test agents for eliciting sweating.

The whole body of the animal except the head and the hind foot or feet to

be tested was put into a bag made of strong cotton cloth, and held by an assistant in a posture convenient for observation. The hairs around the hairless pads were cut as short as possible and the pads were cleaned with a toothbrush and soap-water. Unduly strong rubbing of the pads induced occasionally sweating *per se*. Therefore, about 1 to 3 hours were allowed to elapse before the actual experiment.

The solutions of each test agent were prepared in 0.9% NaCl solution in graded concentrations, and usually 0.05 ml, occasionally 0.1 ml, of each solution was injected subcutaneously into the pads usually from the front with a syringe with a hypodermic needle, the same amount of 0.9% NaCl solution being injected as a control. Usually, the same solution was tested at the same time on any one of the toe-pads and on the central pad (mid pad) of one hind paw. The experiments were repeated at intervals of 1, 2 or 3 days for one month or more after the sciatic nerve section.

In addition, the effects of dihydroergotamine, atropine and procaine on the sweat response to adrenaline and those of procaine on the responses to nicotine and mecholyl were studied. Further, an attempt was made to discriminate the sweat response to direct action of nicotine from the response to its axon reflex provoking action, by using the band method which was described in a previous paper (Wada, Nakamura, Hatanaka and Aoki[31]).

In another series of experiments, adrenaline was injected into the femoral artery or vein, instead of being applied to the pads. The preparation of the blood vessel was made at the time of the sciatic nerve operation; a loop of silk thread was placed conveniently around the exposed vessel to make injection and left there during the whole period for which observations were continued. About 1 to 2 hours after the sciatic nerve section, adrenaline solution in 10^{-3} or 10^{-4} was injected directly into the blood vessel with a syringe with a very small hypodermic needle. Control injections were made with 0.9% NaCl solution. At the time of injection, the hind limb of the side to be injected was exposed from the bag into which the animal had been put in the manner described above.

The magnitude of responses of the foot-pads of both hind feet to adrenaline thus systemically injected was evaluated by counting the number of sweat spots which appeared on each pad and by determining the duration of the response by the procedure described above.

The experiments were repeated in each cat at intervals of 2, 3, 5 and 7 days, until the response to adrenaline became scarcely detectable. A light ether anaesthesia was used when the wound was re-opened 1 to 2 hours prior to the experiment.

All observations were made at environmental temperature of 5° to 28°C and relative humidity of about 50 to 90%.

The following drugs were used: l-adrenaline hydrochloride (Sankyo), nicotine (Merck), mecholyl chloride (Merck), procaine hydrochloride (J.P.), dihydroergotoamine methanesulphonate (Sandoz) and atropine sulphate (J.P.).

It may be mentioned that the first series of experiments were carried out by one of us (N.).

Results

1. *Effects of denervation on the responsiveness of the cat's sweat glands to adrenaline, nicotine and mecholyl locally applied*

Twelve cats were tested for the responsiveness of the denervated sweat glands of the hind paws to both adrenaline and nicotine. The tests with each agents were alternately carried out at intervals of 1, 2 or 3 days after the sciatic nerve section. In 8 of these cats, 13 to 32 days were allowed to elapse between the nerve operations of both sides, and in the other 3 cats the nerve section of both sides was performed at one sitting. Observations were continued for 36 to 54 days in these 11 cats after the nerve section of each side. In another cat nicotine alone was tested on the hind paw of one side until the 15th day after the sciatic nerve section.

In young cats the magnitude of the sweat response to local injections of the same effective concentrations of adrenaline was almost the same for all foot-pads of any one hind foot. In some older cats the response on the middle part of the central pad as well as the inner and outer toe-pads was more evident than that on two mid toe-pads. This difference seemed to be due to the cornification of the pads. In such cases, the minimum effective concentrations of adrenaline were measured mainly on these three pads. The effect of adrenaline on each toe-pads was checked by comparison with that on the middle part of the central pad of the same paw.

The minimum effective concentrations of adrenaline which were determined on the first day of denervation varied in different individuals, but were usually almost identical on both hind feet of the same individuals in which both sciatic nerves had been sectioned at one sitting. The threshold concentrations of adrenaline measured some hours after denervation were 10^{-11} to 10^{-5}. With these concentrations, the sweat spots became visible within 1 to 5 minutes after local injection of 0.05 ml of adrenaline solutions and were counted as 10 to 17 in number. In all of the animals tested the effects of denervation on the sweat glands were found to be consistent. The section of the sciatic nerve on one side was followed by an increased sensitivity of the glands of the homolateral side to adrenaline, as illustrated in Fig. 2. Usually the responsiveness to adrenaline reached its maximum on the 6th to 11th day after operation: the threshold concentrations were lowered to 10^{-13} to 10^{-9}. However, in 2 cats the maximum in the sensitivity was exhibited as early as 2 days after operation. The threshold concentrations were lowered usually to 1/100 to 1/100,000, but rarely to 1/10 of those on the first day of denervation. The period of the hypersensitivity varied from animal to animal, but about 2 to 3 weeks after operation it was replaced by the period of decreased sensitivity. About

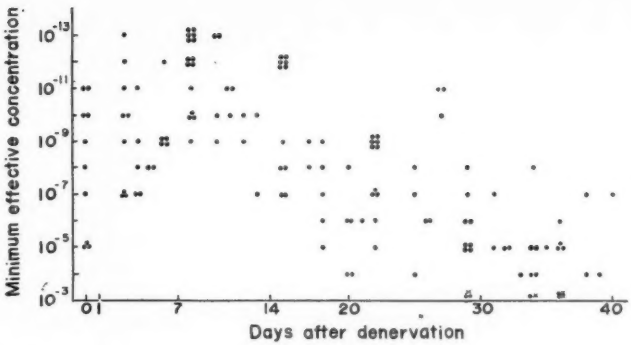

Fig. 2. Changes in the responsiveness of the cat's sweat glands to adrenaline injected subcutaneously into the foot-pads after denervation.
× : no response.

1 to 1½ month after denervation the threshold concentrations of adrenaline were raised up to 10^{-5} to 10^{-3}. In 3 of the cats tested, the sweat response was no longer elicited even with 10^{-3} adrenaline. In some cases in which the sensitivity began to increase again after it had reached the lowest level and spontaneous sweating appeared, the regeneration of the sciatic nerve was verified by either biopsy or autopsy. The effect of re-excision of the regenerated sciatic nerve on the responsiveness of the glands to adrenaline was studied in two cats respectively 39 and 96 days after the previous nerve operation; the supersensitivity similar to that observed after the previous operation could be reproduced, although its magnitude was much smaller.

In the three cats in which the sciatic nerves of both sides had been excised at the same time, the changes in the minimum effective concentrations of adrenaline ran a parallel course and were almost equal for the foot-pads of both sides. In other 8 cats in which 13 to 32 days were allowed to elapse between the nerve operations of both sides, the usual supersensitivity was produced on the recently denervated side, while the sensitivity of the glands of the early denervated side was more or less reduced. During the period of the supersensitivity, a sweat response was produced sometimes even by local injections of 0.9% NaCl solution. But it was discriminated from the response elicited by effective concentrations of adrenaline in its small extent and short duration. The sweat response to the minimum effective concentrations of adrenaline continued for 20–40 minutes in most cases and rarely for 50–60 minutes during the period of supersensitivity.

Further, the effects on the sweat response to adrenaline of dihydro-

ergotamine and atropine were studied in 3 cats and that of procaine in other 5 cats over the period of 2–42 days after denervation. Dihydroergotamine was tested at concentrations of 10^{-5} and 10^{-4}, and atropine at 10^{-7}, 10^{-6} and 10^{-5}. Injections of adrenaline in the threshold concentrations and in ten or one hundred times higher concentrations were made into the foot-pads in mixture with dihydroergotamine or atropine. The results obtained were consistent. The sweat responses to the above described concentrations of adrenaline were abolished by 10^{-4} dihydroergotamine in all tests. With 10^{-5} dihydroergotamine, the sweating by adrenaline was usually totally, but rarely incompletely inhibited. In contrast, atropine had no inhibitory effect on the sweat response to adrenaline. Procaine in 10^{-3} was tested in the similar manner, but it did not interfere with the adrenaline sweating. From these results, it is certain that the sweat responses to the locally applied adrenaline were elicited by its direct action on the denervated sweat glands.

The changes in the responsiveness of the denervated glands to nicotine were quite analogous to those observed with adrenaline. The threshold concentrations of nicotine determined on the first day of the sciatic nerve section were 10^{-11} to 10^{-5}. The supersensitivity to nicotine developed after the denervation; the threshold concentrations reached their lowest level such as 10^{-13} to 10^{-9} on the 6th to 14th day after operation, as illustrated in Fig. 3. The rates of the lowering of the threshold concentrations were

Fig. 3. Changes in the responsiveness of the cat's sweat glands to nicotine injected subcutaneously into the foot-pads after denervation.
× : no response.

approximately identical to those found with adrenaline. The period of

the supersensitivity was usually replaced by the period of the hyposensitivity within two weeks after operation. The decrease in the responsiveness to nicotine was found to be very considerable about 1 to $1\frac{1}{2}$ month after denervation: the threshold concentrations were 10^{-4} to 10^{-2} in most of the test animals. In some animals, the sweat response was no longer obtained even with 10^{-2} nicotine during this period. Sometimes a secondary increase in the response appeared concurrently with the regeneration of the sciatic nerve.

In a previous paper (Wada *et al.*[31]), it was described that in the cat nicotine provokes sweat response by two different mechanisms, that is, by acting directly on the sweat glands and by axon reflex. The response by the latter mechanism differs from that by the direct action of nicotine in that it is elicited by relatively high concentrations of nicotine such as 10^{-5} and 10^{-4} on the first day of denervation and progressively reduced by degeneration of the postganglionic sympathetic nerve. In the present experiments the observations were repeated with the band method on 3 cats. In these animals the sciatic nerves of both sides were sectioned at an interval of 5–17 days. On the first day of denervation, the concentrations of 10^{-5} to 10^{-3} of nictoine were all effective in eliciting the axon reflex sweating: the sweat response spread beyond the band applied transversely to the toe-pad with strong tension to the uninjected side, in spite of the fact that diffusion of the nicotine injected to the side distal to the band was prevented by the band. The axon reflex response was no longer obtained on the 5th to 8th day after denervation. Thus, these results are in good agreement with our previous observations on the axon reflex sweating by nicotine. In contrast, the sweat response produced by direct action of nicotine was still found to be confined to the injected side of nicotine after abolition of the axon reflex; nicotine in 10^{-7} to 10^{-3} was still effective on the 8th to 33rd day after denervation.

It was found that in the cat's toe-pads procaine in 10^{-3} was sufficient to block totally the axon reflex sweat response to potassium cyanide (Wada, Aoki and Koyama[29]) and to local faradic stimulation (Hanawoka[9]), by acting on the receptors responsible. This concentration of procaine is also sufficient to abolish the axon reflex sweating produced by nicotine in 10^{-5} to 10^{-4} in the human skin by acting on the receptors (Wada, Arai, Takagaki and Nakagawa[30]; Wada[28]). Therefore, this concentration of procaine was tested for the effect on the sweating produced by direct action of nicotine in 4 cats over a period of 18 to 23 days after the sciatic nerve section. But the responses to nicotine in the threshold concentrations and in ten or one hundred times higher concentrations were not interfered with by procaine in all tests.

Further, the effect of denervation on the responsiveness to mecholyl

was studied on the hind paws of both sides in 4 cats and on that of one side in 3 cats. The increase in the sensitivity of the glands following the denervation was obtained also from the tests with mecholyl, as presented in Fig. 4.

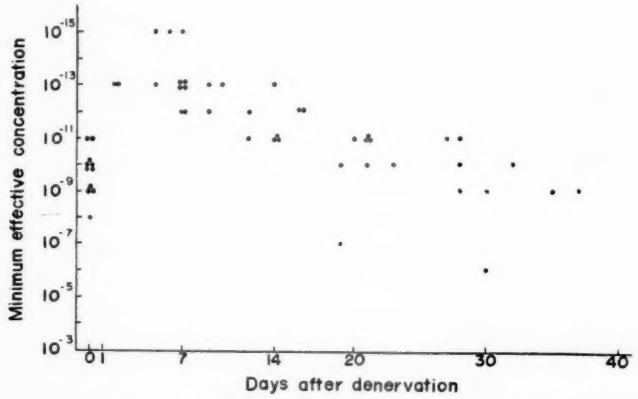

Fig. 4. Changes in the responsiveness of the cat's sweat glands to mecholyl injected subcutaneously into the foot-pads after denervation.

The threshold concentrations of mecholyl were lowered to 10^{-13} to 10^{-12}, rarely to 10^{-15}, on the 6th to 8th day after denervation, in contrast to 10^{-11} to 10^{-8} on the first day: the threshold concentrations decreased to 1/100 to 1/1,000,000 of those on the first day of operation. About one month after denervation the responsiveness to mecholyl was found to be more or less reduced, as compared with that on the first day, but some paws exhibited no reduction of the responsiveness: the minimum effective concentrations were 10^{-11} to 10^{-6}.

In 4 other cats the effect of procaine on the sweat response to mecholyl in the threshold concentrations and in ten or one hundred times higher concentrations was studied at various intervals for a period of 28 to 31 days after denervation: the sweating by the threshold concentrations of mecholyl was very often inhibited by procaine in 10^{-3}, but the responses to the higher concentrations of mecholyl were not affected by this concentration of procaine.

2. *Effect of denervation on the responsiveness of the cat's sweat glands to adrenaline systemically applied*

The experiments in this series were carried out on 10 unanaesthetized cats with both sciatic nerves sectioned at one sitting. In 5 cats, 0.2 ml of 10^{-3} adrenaline (0.07–0.09 mg per kg of body weight) was injected directly

into the femoral vein. On the first day after denervation, no sweat response was produced by this dose of adrenaline in 3 of the 5 cats. In the other 2 cats, the injections of adrenaline caused a definite but slight response on the first day. In general, the magnitude of the response, namely, the number of sweat spots and duration of the sweating varied from pad to pad. In some toe-pads sweat spots were scarcely perceptible. When the response was produced to a small extent in the central pad, it was localized usually to its middle part only. The response became more evident on the 3rd day and its magnitude reached a maximum in all foot-pads on the 8th or 10th day after denervation. Not rarely, the sweat spots counted over 100 on one toe-pad and over 350 on one central pad involving its two side eminences.

A similar increase in the response was observed in the other 5 cats in which 0.1 ml of 10^{-4} or 10^{-3} adrenaline (0.003–0.06 mg per kg of body weight) was injected into the femoral artery of one side. In all of these animals the sweating occurred in 1 to 3 foot-pads of the injected side alone on the first day of the sciatic nerve section. This response ceased usually within 10 to 20 minutes after injection. On the 4th or 6th day, the response began to extend not only over all foot-pads of the injected side, but also over some of the pads of the uninjected side. The maximum increase in the magnitude of the response was found by the tests performed on the 8th day after operation. Usually the response on the injected side was more remarkable than that on the uninjected side. In one case the response persisted for about two hours on the paws of both sides.

After reaching its maximum, the magnitude of the response to adrenaline decreased progressively, and sweating was no longer produced by the same doses of adrenaline on the 17th to 22nd day after denervation in four animals tested with the intravenous adrenaline, and on the 15th to 22nd day in three animals tested with the intra-arterial adrenaline. However, even in this period, the denervated sweat glands responded well when the doses of adrenaline were increased.

In this series of experiments, it was difficult to evaluate precisely the changes in the responsiveness of the denervated sweat glands to adrenaline, but the period of the supersensitivity of the denervated glands could be judged to be approximately 10–17 days following the denervation. This is fairly in harmony with the results obtained from the preceding series of experiments with local injection of adrenaline. Therefore, this exaggeration of the response to adrenaline applied systemically may be due to the increased responsiveness of the sweat glands themselves. Further, this period of the exaggeration of the response to the systemic administration of adrenaline is almost identical with that of the increased response to pilocarpine which was described by Burn.[1] It may be noted that there

was no relation between the changes in the magnitude of the responses and those in the body weight of animals during the whole period of observations.

Discussion

The present results indicate that the minimum effective concentrations of adrenaline, nicotine and mecholyl for eliciting the sweat response in the foot-pads of cats were lowered early and raised late after denervation of the sweat glands by section of the peripheral nerve. This finding is in keeping with the increased response to pilocarpine which was occasionally observed in cats by Burn[1] with ocular inspection, and by Simeone, Mentha and Rodrigues,[25] using the electrical skin resistance method. The supersensitivity persisted usually for 2 to 3 weeks. This evidence is compatible with Cannon's law of denervation. Burn[1,2] applied pilocarpine subcutaneously to the flank for testing the effect of degenerative section of the sympathetic nerve on the sweat response in the foot-pads, and explained the increased response to pilocarpine as being due to the removal of the sympathetic control of the blood vessels. This explanation may be applied also to the results obtained by Simeone *et al.*[25] in the experiments with intravenous injection of pilocarpine. The experiments of Burn[2] on some kittens, however, suggested that the denervated glands themselves became supersensitive to pilocarpine. The possibility that circulatory changes after denervation influence the sweat response of the denervated sweat glands was reduced in the present experiments to a minimum by local injection of the test agents to the foot-pads. Therefore, the results obtained may be due largely, though not solely, to the changes in the excitability of the denervated glands themselves.

In the present experiments, the early increase in the responsiveness is more outstanding than the late decrease at least during the observation period of one month and a half. The maximum of the supersensitivity to all adrenaline, nicotine and mecholyl was observed usually on the 6th to 11th day after denervation. Similar findings on the development of the supersensitivity to adrenaline have been reported on the cat's eye (6th to 10th day, by Sugawara[26]) and on the cat's nictitating membrane (7th to 10th day, by Hampel[8]) after deprivation of the postganglionic sympathetic nerve supply. Therefore, it seems to us that there is no essential difference in the development of supersensitivity between the sympathetic cholinergic system and sympathetic adrenergic system. Further, it must be noted that there was no specificity of the sensitization of the denervated sweat glands to stimulating agents, in so far as it was studied with adrenaline, nicotine and mecholyl.

The decrease in the responsiveness to the test agents which occurs after the period of the supersensitivity would be analogous to that which develops early after denervation in the human sweat glands. Denervation of the human sweat glands by postganglionic sympathectomy or by the peripheral nerve section produces decrease, instead of increase, in their sensitivity to acetylcholine (Rothman and Coon[23]; Kahn and Rothman[13]; Janowitz and Grossman[11]; Chalmers and Keele[4]), to mecholyl (Kahn and Rothman[13]; Randall[21]), to pilocarpine (Coon and Rothman[5]; Kahn and Rothman[13]; Janowitz and Grossman[11]) and to adrenaline (Janowitz and Grossman[12]; Takagaki, Goto and Mishina, personal communication), when tested with intradermal injections.

Luchsinger[17] found that the sweat response in the cat's sweat glands diminished progressively after section of the sciatic nerve. This observation was confirmed by Nawrocki,[18] Vulpian,[27] Ott[19] and Langley.[15] In the experiments of these investigators, the decrease in the sweat response to pilocarpine was recognized two days to about two weeks after denervation. Such early hyposensitivity to pilocarpine was occasionally observed by Burn[1] and Simeone *et al.*[25] A similar finding was obtained also by Kahn and Rothman[13] on the sweat response to acetylcholine. In one of their three cats, however, the sweat response to acetylcholine showed an increase for the first 24 hours after denervation, followed soon by a decrease. In the present experiments with adrenaline, nicotine and mecholyl, however, we found no evidence of decrease in the responsiveness to these agents in the eary stage of denervation. We have no explanation for this discrepancy at present.

In a previous paper (Wada *et al.*[31]), it was demonstrated that nicotine elicits the sweat response in the foot-pads of the cat by its direct action on the sweat glands and by an axon reflex. This was confirmed by the present experiments. The axon reflex sweating by nicotine is abolished within about a week after sympathetic ganglionectomy or after section of the peripheral nerve, whereas the sensitivity of denervated sweat glands to the direct action of nicotine increases to a maximum at the end of about a week, as described above. This provides a strong support for the view that the axon reflex sweat response depends on the integrity of the sympathetic sweat nerve fibres.

Beginning with the work of Dieden[6] in 1916, a number of investigators, using ocular inspection for detecting sweat secretion, have failed to obtain any definite evidence of the stimulating action of adrenaline on the cat's sweat glands (Langley[15]; Langley and Uyeno[16]; Schilf and Mandur[24]; Peserico and Stella[20]; Burn[2]; and Kuré, Okinaka, Maéda and Kato[14]). On the contrary, Fröhlich and Zak,[7] using the method of Jürgensen, found that adrenaline injected subcutaneously or locally to the pads of the hind

paw elicited sweat response even after section of the sciatic nerve; but this response was not abolished by ergotamine. In the same year, Hara[10] reported that the skin galvanic reaction attributable to sweat secretion was produced by systemic administration of adrenaline in the hind paw which had been denervated by section of the sciatic nerve. Coon and Rothman occasionally observed minimal, but not regularly reproducible, sweating on the cat's foot-pads after intradermal injection of adrenaline (Rothman[22]). However, these findings seem insufficient to dispel the general belief that adrenaline is devoid of a stimulating action on the cat's sweat glands. Contrary to this belief, the present experiments demonstrated that adrenaline, injected locally or systemically, elicits the sweat response in the foot-pads of the cat which is abolished by dihydroergotamine, but not by atropine.

Summary

1. The effects of denervation of the cat's sweat glands on their responsiveness to adrenaline, nicotine and mecholyl were studied by measuring the minimum effective concentrations of these agents for eliciting the sweat responses when injected subcutaneously into the foot-pads.

2. The sensitivity of the sweat glands to all these test agents was increased by denervation, its maximum being reached at the end of about a week after denervation. The supersensitivity persisted usually for about 2 to 3 weeks, after which the sensitivity diminished more or less progressively. In general, the supersensitivity was a more regular and more certain effect of the denervation than the hyposensitivity at least for the observation period of about one month and a half after denervation.

3. Adrenaline was proved to have the property of eliciting the sweat response in the cat's foot-pads when applied locally or systemically. This response was abolished by dihydroergotamine, but not by atropine and procaine.

4. The sweat responses to intravenous and intra-arterial injections of adrenaline were exaggerated for a certain period following the denervation. This may be explained as being due to the increased sensitivity to adrenaline of the denervated sweat glands themselves.

We wish to express our thanks to Professor Masao Wada for his guidance and advice throughout this work.

References

1) Burn, J. H., J. Physiol., 1922, **56**, 232–247.
2) Burn, J. H., *ibid.*, 1925, **60**, 365–378.
3) Cannon, W. B., Am. J. Med. Sci., 1939, **198**, 737–750.
4) Chalmers, T. M. & Keele, C. A., Brit. J. Dermatol., 1952, **64**, 43–54.

5) Coon, J. M. & Rothman, S., J. Pharm. & Exp. Therap., 1941, **73**, 1–11.
6) Dieden, H., Z. Biol., 1916, **66**, 387–390.
7) Fröhlich, A. & Zak, E., Arch. exp. Pathol. u. Pharmakol., 1932, **168**, 620–637.
8) Hampel, C. W., Am. J. Physiol., 1935, **111**, 611–621.
9) Hanawoka, N., Japanese J. Physiol., 1958, **8**, 114–122.
10) Hara, Y., Jap. J. Med. Sci., Biophysics, 1932, **2**, 215–247.
11) Janowitz, H. D. & Grossman, M. I., J. Invest. Dermatol., 1950, **14**, 453–458.
12) Janowitz, H. D. & Grossman, M. I., Experientia, 1951, **7**, 275.
13) Kahn, D. & Rothman, S., J. Invest. Dermatol., 1942, **5**, 431–444.
14) Kuré, K., Okinaka, S., Maéda, S. & Kato, H., Pflügers Arch., 1936, **237**, 40–53.
15) Langley, J. N., J. Physiol., 1922, **56**, 110–119.
16) Langley, J. N. & Uyeno, K., *ibid.*, 1922, **56**, 206–226.
17) Luchsinger, B., Pflügers Arch., 1877, **15**, 482–492.
18) Nawrocki, F., Centralbl. f. d. med. Wiss., 1878, 97–99.
19) Ott, I., J. Physiol., 1879, **2**, 42–65.
20) Peserico, E. & Stella, G., Arch. di fisiol., 1923, **21**, 419–426.
21) Randall, W. C., J. Clin. Investig., 1946, **25**, 761–767.
22) Rothman, S., Physiology & biochemistry of the skin, Univ. of Chicago Press, 1954 (p. 166).
23) Rothman, S. & Coon, J. M., J. Invest. Dermatol., 1940, **3**, 79–97.
24) Schilf, E. & Mandur, I., Pflügers Archiv., 1922, **196**, 345–357.
25) Simeone, F. A., Mentha, C. & Rodrigues, H. A., Am. J. Physiol., 1951, **165**, 356–364.
26) Sugawara, T., Tohoku J. Exp. Med., 1927, **8**, 355–404.
27) Vulpian, A., C. R. Acad. Sci., 1878, **87**, 311–313.
28) Wada, M., J. Invest. Dermatol., 1954, **23**, 63–66.
29) Wada, M., Aoki, T. & Koyama, W., Experientia, 1958, **14**, 102–103.
30) Wada, l, Arai, T., Takagaki, T. & Nakagawa, T., J. Appl. Physiol., 1952, **4**, 745–752.
31) Wada, M., Nakamura, Y., Hatanaka, K. & Aoki, T., Arch. internat. Physiol. Biochim., 1955 **63**, 203–212.
32) Wada, M. & Takagaki, T., Tohoku J. Exp. Med., 1948, **49**, 284.

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Interference and Diffraction of Spreading Induction

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Introduction

In previous papers[1),2),3)] by Motokawa *et al.* it was shown that spreading induction was reflected and refracted at the boundary between dark- and light-adapted parts of the human retina. The laws of reflection and refraction were shown to apply well to this physiological phenomenon. A light-adapted retinal area prismatic in shape served to change the direction of propagation of spreading induction, and the refractive index of the area was measured in this experiment. On the other hand, the refractive index was computed from the propagation velocities of spreading induction in the dark-adapted and the light-adapted retinal areas. The values obtained from these different experiments were found to agree very satisfactorily. From this agreement it was inferred that the spreading induction might be a kind of waves.

In the present investigation interference and diffraction of spreading induction were studied with a view to providing more conclusive evidence for its wave-nature.

Experimental

Method

The general principle of the method for initiating and detecting the spreading induction was the same as described in the previous papers.[1),3)] A pair of an inducer and a detector was used. They were always complementary to one another. The arrival of spreading induction at the detector was tested by a white test light followed by an electric stimulus of 0.1 second. The indirect induction preestablished around the detector disappeared when the spreading induction caused by the inducer arrived at the detector. This phenomenon is termed neutralization of retinal induction. Neutralization is usually limited to the part of the detector at which the spreading induction arrived.

The inducer and the detector were presented on a vertical screen at a distance of 30 cm. in front of the subject's eye. On the way from the inducer to the detector a grating of alternate light and dark stripes of equal width was presented. The spreading induction initiated by the inducer had to traverse the after-image of the grating to reach the detector. If the grating was effective, it was to be expected that neutralization would occur in a manner similar to an optical diffraction pattern.

In order to survey the manner of neutralization at the detector, a white test light, a spot of 1 mm. in diameter, was presented at various points along the detector, and the contrast effect (C.E.) at each point was measured after the method described in the previous paper. The C.E. represents the difference between the electrical excitability (the reciprocal of threshold) obtained with a sequence of stimuli, colored and white, and that obtained with the white test light alone. A zero-value of C.E. is an indication of complete neutralization. The details of experimental conditions for each experiment will be mentioned together with the results.

Results

The most primitive experiments of diffraction

The arrangement of patches and the order of their presentation are shown in the inset of Fig. 1. The detector, D, was yellow in hue and its intensity of illumination was 330 lux without a yellow gelatin filter. The inducer, I, was blue in hue and its illumination was 3300 lux without a blue filter. The screen, Sc, was a white patch for preventing leakage of spreading induction. The patch, G, provided a slit through which spreading induction passed towards the detector in experiment A. In the other experiments, B and C, it represented a grating consisting of light and dark stripes. The illumination of G had to be low to avoid irradiation. Otherwise the retinal image of G would be blurred owing to irradiation and could not serve as a grating. The illumination of G was only 12 lux. A fixation point, F, was placed at a shorter distance from G so that a sharp image of G might be formed on the retina.

With this set of inducer and detector the electric stimulus was delivered at 1.5 seconds from the end of the white test light, Wh.

Patterns of neutralization along the detector are shown in Fig. 1. In experiment A neutralization occurred only at the middle part of the detector. The spreading induction initiated by the inducer must have run a straightforward course to reach the middle part of the detector. In experiment B the grating, G, consisted of two dark slits of 1 mm. in width separated by a white line of the same width. If the two beams of spreading induction coming through both slits of G were to run independent straight-

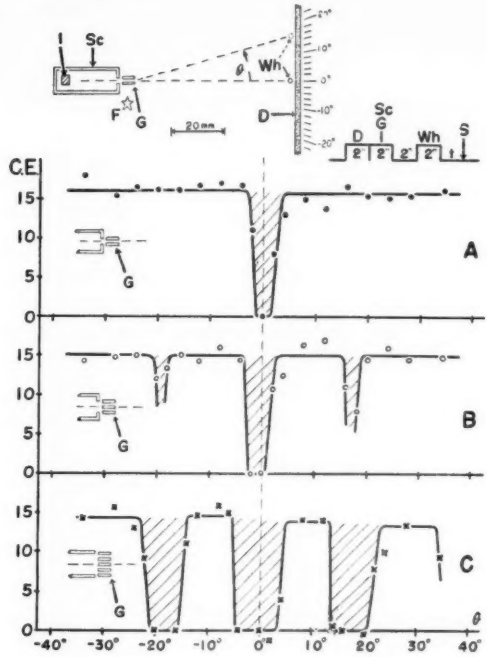

Fig. 1. Diffraction of spreading induction as revealed by patterns of neutralization. Ordinates: Contrast effects (C.E.). Abscissae: Directions of spreading induction in degrees. Patches used and temporal sequence of their presentation are shown in insets. D detector, F fixation point, G slit or grating, I inducer complementary to D, Sc screen, S electric stimulus of 0.1 sec., t time interval between white test light, Wh, and S. Neutralized parts of detector are shaded.

forward courses, neutralization would occur at two parts of the detector. In reality, however, neutralization occurred at three parts, as can be seen in Fig. 1 B. In this case neutralization is complete at the middle part of the detector, but incomplete on both sides of it. Such a pattern may be accounted for most easily in terms of diffraction. If this interpretation is correct, a similar but more conspicuous pattern should be obtained by increasing the number of elements of the grating. In experiment C the number of elements was made 4, that is, twice as great as that in experiment B. The pattern of neutralization obtained in this experiment was really similar to that in experiment B, but the neutralization at the sidebands was far more conspicuous. The fact that the positions of neutralization depended little on the number of elements of the grating is a characte-

ristic of diffraction. The parts at which neutralization occurred correspond with light bands of an optical diffraction spectrum, and the parts at which no neutralization occurred correspond with dark bands. Therefore the term "dark and light bands" will, for simplicity, be used also for description of our phenomenon.

Experiments with a set of patches imitating a simple diffraction grating

With the set of patches illustrated in Fig. 1 conspicuous diffraction patterns were obatained only when the illumination of the inducer was sufficiently strong. This was due to the circumstance that only a small fraction of the spreading induction produced by the inducer could reach the detector. To increase the number of elements of the grating would have been profitable, but it was difficult to do this, because the grating would have become too large in size.

In the following experiments the number of elements was limited to 4, but instead, we tried to concentrate diffracted spreading induction by the use of a lens-shaped patch.

The arrangement of patches was as in Fig. 2. A lens-shaped patch,

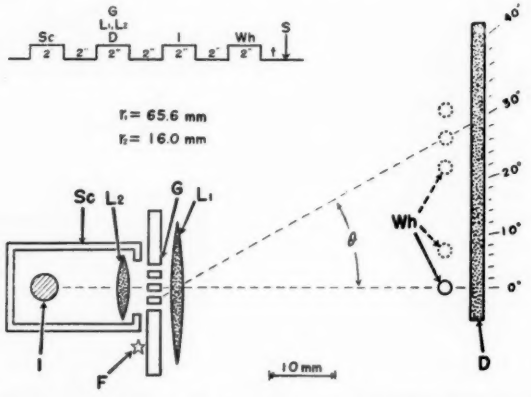

Fig. 2. Set of patches imitating a simple diffraction grating. L_1 and L_2: Lens-shaped patches of the same color as detector, D. r_1 and r_2 represent radius of curvature of L_1 and that of L_2 respectively. Other notations were the same as in Fig. 1.

L_1, served to focus diffracted spreading induction to the detector. Another patch, L_2, was a device to make parallel beams of the spreading induction from the inducer. These patches were of the same color as the detector and complementary to the inducer. Under this condition the refracive index of the lens-shaped image of the patch is highest, as was shown in the previous paper.[3] The radius of curvature of L_1 was calculated on

the basis of the formula for thin lenses, using refractive index 1.82 and distance between the lens and the detector. The illumination for D, I, Sc and Wh was 70 lux without filters.

In order to work with different kinds of spreading induction, red, yellow, green and blue inductions were produced by a blue-green, a blue, a purple and a yellow inducer respectively. The interval between the end of white test light and the elektric stimulus was 1, 1.5, 2 and 3 seconds respectively.

The results obtained are summarized in Fig. 3. As can be seen in this figure, neutralization occurred at two or three parts of the detector. They would correspond with the light bands of the zero-th, the first and the second order of an optical diffraction spectrum obtained by a simple grating. It is worthy of note that the angular distance between two consecutive light bands are different depending upon the kinds of spreading induction; it is shortest for the red spreading induction, becoming greater in the order: yellow, green and blue.

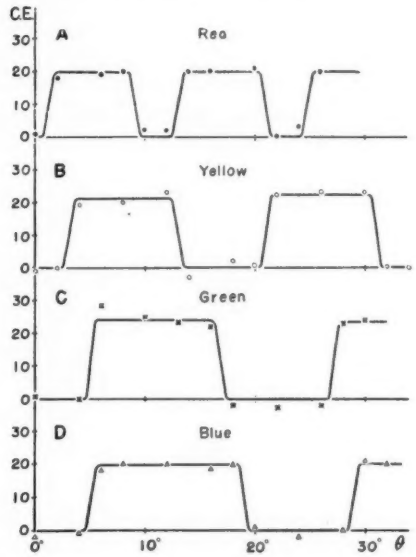

Fig. 3. Diffraction patterns obtained with arrangement of patches shown in Fig. 2. A, B, C and D refer to red, yellow, green and blue spreading inductions respectively.

The formula for a simple diffraction grating is as follows:

$$d \sin \theta = m\lambda \quad [m=0, 1, 2, \ldots\ldots] \quad (1)$$

where d and λ denote the constant of the grating, i.e. the sum of the width of a light line and that of a dark one, and the wave-length respectively. If this formula holds for the present experiments, the wave-length of each kind of spreading induction can be computed as in the following example: For the red spreading induction values of θ may be obtained from the uppermost curve in Fig. 3. They are 11° for the light band of the first order (m=1), and 23° for that of the second order (m=2). The constant of the grating was 2 mm. Therefore the following relations hold:

$$2 \sin 11° = \lambda \quad [m=1] \quad \text{and}$$
$$2 \sin 23° = 2\lambda \quad [m=2].$$

From these relations two values 0.392 and 0.391 mm. were obtained for the wave-length of the red spreading induction. These values are in accordance with each other, and such satisfactory agreement indicates the validity of formula (1) for our phenomenon. The values obtained above refer to the visual field at a distance of 30 cm. in front of the eye. They can be reduced to the values in the retina by multiplying them with a factor 1.7/30, where the numerator 1.7 represents the posterior nodal distance of the eye.

In the same way the wave-lengths of the other kinds of spreading induction were obtained and represented in Table I. As can be seen in

TABLE I

Wave-lengths of Spreading Induction Determined by Principle of Simple Diffraction Grating

Hue of inducing light	Character of spreading induction	Wave-lengths	
		In visual field 30 cm. from eye (mm.)	In retina (μ)
Blue-green	Red	0.382	21.7
Blue	Yellow	0.585	33.1
Purple	Green	0.749	42.5
Yellow	Blue	0.813	46.2

this table, the wave-length becomes longer as in the order: Red, yellow, green and blue. It is to be noted that this order is reverse to the order of spectral lights concerning wave-lengths.

Experiments based on the principle of Rowland's concave grating

In further experiments an arrangement of patches imitating Rowland's concave grating was used. The patches are illustrated in Fig. 4. In this system spreading induction reaching the detector was great in amount compared with the systems mentioned above, because the number of ele-

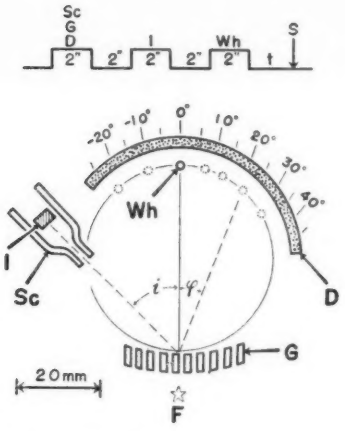

Fig. 4. Set of patches imitating Rowland's concave grating. D detector, F fixation point, G grating, I inducer, Sc screen, S electric stimulus and Wh white test light. I and D were complementary in hue to each other.

ments was greater, and reflection instead of transmission at the grating was utilized. As a consequence even weak spectral light could be used for the inducer. With this set of patches clear-cut results were obtained even from untrained subjects.

The diffraction patterns illustrated in Fig. 5 were obtained from three different subjects who were not so well-trained as the subjects in the above-mentioned experiments. All of them were beginners in this kind of experiment. The inducer was blue in hue so that the spreading induction caused by it was yellow in character. In these experiments light bands were greater in width than dark bands so that calculation of wave-lengths were carried out on the basis of dark bands. For the latter the following formula of Rowland's grating holds:

$$d\,(\sin i \pm \sin \varphi) = (2m+1)\frac{\lambda}{2} \quad [m=0,\ 1,\ 2,\ \ldots\ldots] \qquad (2)$$

From the diffraction patterns illustrated in Fig. 5 and based on this formula, we obtained 31, 29 and 31 μ as the wave-length of yellow spreading induction in the retina for the three subjects respectively. They are satisfactorily consistent with one another. In all these experiments the constant of the grating used was 2 mm.

Next, the constant of the grating was varied, and its effect was investigated upon the diffraction pattern as well as upon the wave-length. The spreading induction used was blue in character. In Fig. 6 are illustrated

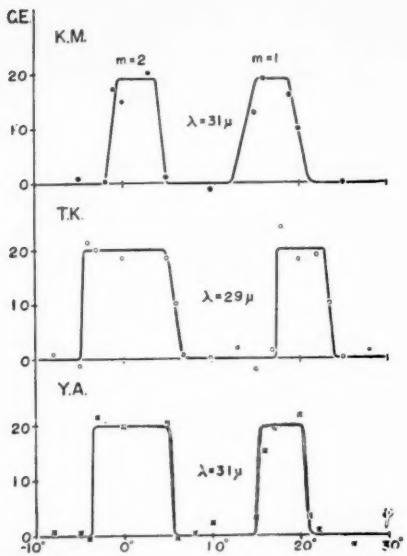

Fig. 5. Diffraction patterns of yellow spreading induction obtained with set of patches shown in Fig. 4 from three different subjects under one and the same experimental conditions. m : Order of dark bands in which no neutralization occurred. Wave-lengths computed from these pattersn are given.

diffraction patterns obtained by Rowland's gratings with three different constants, 2, 3 and 4 mm. from one and the same subject. As can be seen in this figure, the interval between two consecutive dark bands becomes narrower as the constant of grating increases. This property coincides with what can be predicted from formula (2). We computed wavelengths on the basis of these diffraction patterns and obtained 51.2, 49 and 50.7 μ. These values are satisfactorily consistent with one another, although they were obtained from the diffraction spectra so different in form.

Discussion

It was shown above that diffraction of spreading induction occurred following the same principle as that of light. Both principle of a simple diffraction grating and that of Rowland's grating were applicable to our phenomenon, and consistent results were obtained by these methods from different individuals. This fact may be regarded as evidence for the undulatory nature of spreading induction predicted from our previous experiments on reflection, refraction and conduction of spreading induc-

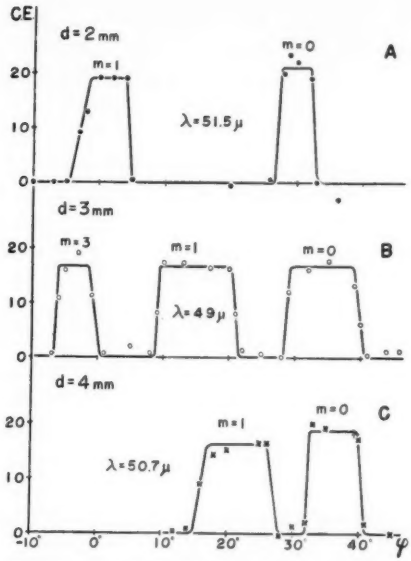

Fig. 6. Diffraction patterns obtained from one and the same subject for different constants of Rowland's grating. Constant, d, was 2, 3 and 4 mm. for experiments A, B and C respectively.

tion. It is noteworthy that the wave-length of spreading induction was found different according to the kinds of it in contrast with the fact that the propagation velocity of spreading induction in the dark-adapted retina depended little on the kinds of spreading induction. This relation resembles the constant propagation velocity of lights of various wave-lengths in vacuum.

There are, however, some difference between diffraction of light and that of spreading induction. For example, the amplitude of a diffraction pattern cannot be a measure of the intensity of spreading induction. And this is based on the characteristic of neutralization that occurs nearly in the manner of all-or-none; spreading induction of any intensity can cause neutralization of the same degree provided that it is above the threshold. This is the reason why the diffraction curves obtained above consisted of trapezoids of the same height.

A part of the findings stated in the present paper was published in Japanese seven years ago.[4] Since then this phenomenon has been confirmed many times on a number of subjects in our laboratory. Especially, experiments with the patches imitating Rowland's grating yielded always clear-cut results.

SUMMARY

It was shown in previous papers that spreading induction was reflected and refracted at the boundary between dark-adapted and light-adpated retinal parts. In the present experiments evidence has been provided that spreading induction shows diffraction. A pair of patches, an inducer and a detector, was used together with a patch whose retinal image served as a diffraction grating. The inducer was a patch to initiate spreading induction, and the detector a devide to detect the arrival of the spreading induction from the inducer. The grating patch consisting of light and dark stripes was presented half-way between the inducer and the detector so that the spreading induction from the inducer had to traverse the retinal image of the grating to reach the detector. Under these conditions the arrival of spreading induction was found only at several discrete parts of the detector in a manner similar to light bands of a diffraction spectrum. An arrangement of patches imitating Rowland's concave grating was found most suitable for this experiment. The wave-length of spreading induction was measured by this method, and values about 30 μ and 50 μ were obtained for yellow and blue spreading inductions respectively.

The wave-length was longer in the order:

red<yellow<green<blue. It is reverse to the order of spectral lights concerning wave-lengths.

References

1) Motokawa, K. & Komatsu, M., Tohoku J. Exp. Med., 1957, **67**, 29.
2) Motokawa, K. & Komatsu, M., *ibid.*, 1958, **67**, 149.
3) Motokawa, K., Ebe, M., Komatsu, M. & Watanabe, K., *ibid.*, 1958, **68**, 139.
4) Motokawa, K., Nisshin-igaku (Jap.), 1951, **38**, 211.

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Flicker and Fusion of Potentials Recorded from Fish Retina with a Microelectrode

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Introduction

Svaetichin[1,2] recorded an intracellular slow potential showing sustained negativity during illumination. Although this potential was at first supposed to originate in a cone, it was doubted by various investigators[3,4,5]. Recently it has been shown by Oikawa, Ogawa and Motokawa[6] that this potential has its origin in a secondary neuron of the retina. MacNichol, Macpherson and Svaetichin, Tomita and Mitarai have reached the same conclusion as Oikawa *et al.* (personal communication).

As to the mechanism of flicker fusion some authors stress the importance of the peripheral mechanism, but others that of the central mechanism. Most of the discussions on this problem were done about the data obtained by the psychophysical method or by the method utilizing the optokinetic phenomenon[7,8,9,10]. Some related experiments have been made with the ERG, but it is not always easy to interpret the flicker fusion of the ERG which consists of a number of components. Svaetichin made some preliminary experiments on the flicker fusion of the slow potential he discovered.

In the present investigation the same phenomenon was studied more systematically with special reference to temperature.

Experimental

Method

Experiments were made on retinae of carps (Cyprinus carpio). The so-called inverted retina was prepared in the manner described by Svaetichin[1] and by Motokawa, Oikawa and Tasaki[4], and was mounted on cotton wool soaked with saline on which an indifferent electrode of Ag-

AgCl-agar type was set. The cotton wool was placed on a flat glass vessel through which warm or cold water was perfused to control the temperature of the preparation. The actual temperature of the retina was measured by means of a thermojunction and a galvenometer.

A KCl-filled microelectrode of about 0.3 μ in diameter was inserted from the receptor side of the retina. For recording of intracellular potentials a direct-coupled amplifier with a cathode-follower input and a cathode-ray oscilloscope were used.

Intermittent light was produced by means of a rotating sector of light-dark ratio 1:1. The rate of flicker was measured by a well-calibrated electric tachometer.

Results

When a microelectrode was inserted to a depth of 140–200 μ, the resting potential showed a sudden change of 20–50 mV. In response to illumination a further negative-going deflection of 10–30 mV was obtained. This slow potential (SP) lasted as long as illumination was continued (see Fig. 1).When the retina was subjected to intermittent illumina-

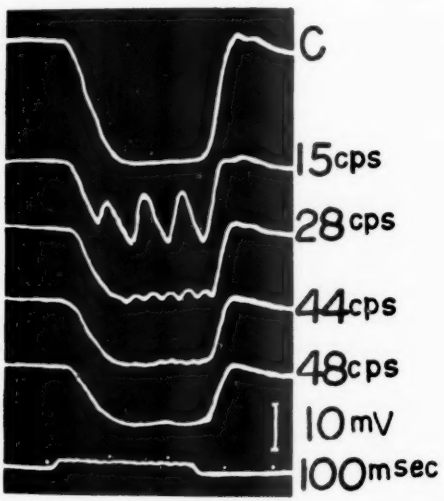

Fig. 1. Flicker and fusion of potential recorded from N-element of carp retina. C: Response to continuous light. Negativity downwards. Temperature 29°C.

tion, ripples were superimposed on top of the SP in exact accordance with the flickering rate. As the frequency of intermittence was increased, the

individual fluctuations on top of the SP were reduced in amplitude to disappear at a certain frequency of flicker. This is a critical fusion frequency (CFF) of the potential. In the example shown in Fig. 1, the temperature of the preparation was kept at 29°C, and the CFF at this temperature was 48 cps.

Motokawa *et al.*[4] showed that there are two types of elements, N and A, in the carp retina. The potential of the N-element is always negative-going, while that of the A-element changes its polarity according to the wave-length of light. In the A-element the response to a continuous white light stimulus shows an overswing towards the positive side at cessation of illumination, but in the N-element such an overswing is either lacking or very slight. The potentials shown in Fig. 1 were recorded from a N-element.

In the present investigation it was found that in general A-elements were capable of responding to flicker of much higher frequency than N-elements. Fig. 2 shows records obtained from an A-element. In this

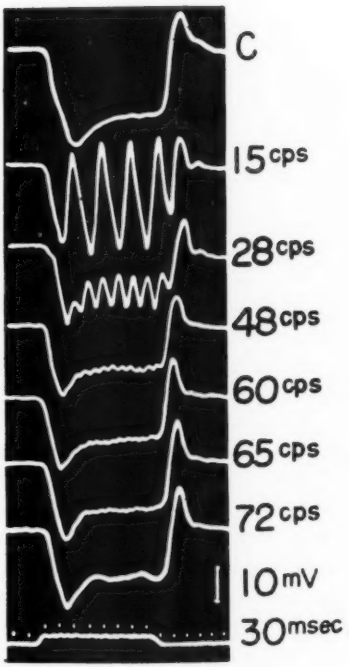

Fig. 2. Flicker and fusion of potential recorded from A-element of carp retina. Temperature 29°C.

experiment the temperature was kept at the same level as in the experiment of Fig. 1. As can be seen in the records, the CFF was 72 cps. which is much higher than a CFF of 48 cps. in the preceding example of N-elements.

The amplitude of each ripple caused by flickering light of a given frequency was much larger in the A-element than in the N-element.

Next, the dependence of CFF on the intensity of illumination was investigated. As there was no qualitative difference between the two types of elements concerning this property, the results obtained from N-elements will be reported. In Fig. 3 CFF—log intensity relations obtained at three different temperatures are illustrated. In these experiments decision of CFF was made by observing the screen of the Braun tube. The intensity of light was varied by neutral filters. At lower and moderate intensities the potential was brought to fusion by changing the intensity of light, but at higher intensities near the maximal CFF, fusion was brought about by increasing the rate of flicker.

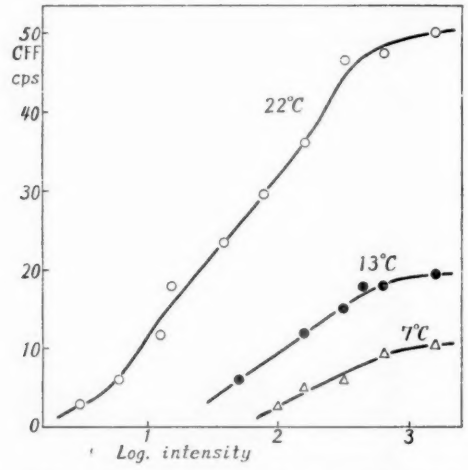

Fig. 3. CFF—log intensity relations of N-element at three different temperatures.

As can be seen in Fig. 3, a linear relation such as stated by the Ferry-Porter law holds in the range of moderate intensities. The relation can be represented by a single curve. At higher intensities the CFF tends to become saturated. The maximal CFF is higher as the temperature is higher. This property does not agree with the property of the CFF-log intensity relation of the sun fish determined by Crozier, Wolf and Zerrahn-Wolf[9] with the behavorial method. The general trend of the curve at each

temperature is very similar to that of the curve from the sun fish. Our curve obtained at 22°C covers about three log units of intensities, while at 13 and 7°C the curves stand on two or one log units.

The above-mentioned change of CFF with temperature was reversible, as is shown by an example in Fig. 4. In this experiment the temperature was raised to about 22°C from the room temperature of about

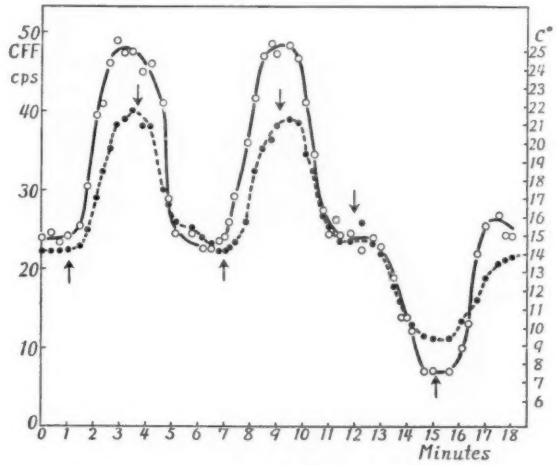

Fig. 4. Experiment showing reversibility of CFF. Continuous curve refers to CFF, and broken one to temperature. Arrows indicate moments at which temperature of perfused water was changed.

14°C and then lowered again to the initial temperature. The same procedure was repeated twice. In the figure the continuous curve connecting open circles and the broken one connecting solid circles refer to the CFF and the temperature respectively. Arrows indicate the moments at which the temperature of the perfused water was changed. It is to be noted that the curve of CFF runs almost parallel to that of temperature, indicating satisfactory reversibility of the phenomenon. Above 26°C reversibility was, however, not so satisfactory.

Discussion

There are two types of elements, N and A, which can be distinguished on the basis of the difference in off-response. As the A-element shows a positive overswing at cessation of illumination, the rate of potential change at "off" is generally higher than that of the N-element in which such an overswing is lacking. This relation seems to be responsible for

the higher CFF of the A-element. In Fig. 5, it is shown how the time course of a single response of a N-element depends on temperature. At lower temperatures the rate of negative swing at "on" is much slower than that of the off-swing. In view of this fact it may be said that the on-swing becomes fuged at a lower frequency than does the off-swing. The determining factor for the CFF must be the off-swing also in the N-element. Therefore it is quite natural that the A-element with a higher rate of the off-swing shows a higher CFF.

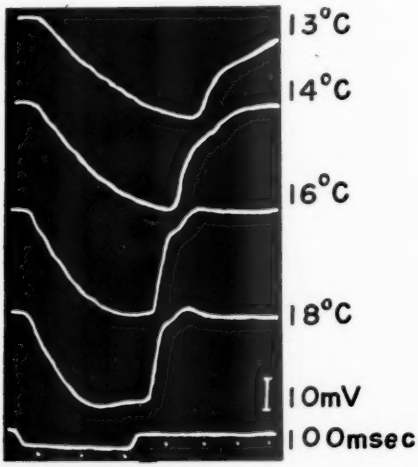

Fig. 5. Time course of response to steady illumination at various temperatures. Period of illumination is marked at bottom.

It is a well-known fact that in the curves of CFF—long intensity determined with the psychophysical and the behavorial methods two portions can be distinguished. The portion standing on the lower intensities is said to be related with the rod function, and that over the higher intensities is regarded as representing the cone function. In other words the cones are believed to have higher CFFs than the rods. In view of the above-mentioned fact that the A-element shows a higher CFF than does the N-element, one may be tempted to correlate the two type of elements with the cone and the rod. However, our knowledge about these elements does not seem to favor this assumption. As was shown by Motokawa *et al.*[4] the spectral distribution of the N-element does not coincide with the absorption curve of porphyropsin which is contained in the rod of the fish retina. Responses of N-elements can be recorded even when the outer segments of rods are removed together with the

pigment epithelium from the retina.

For interpretation of the CFF—log intensity relation two theories have been proposed: (1) the photochemical theory as formulated by Hecht, Shlaer and Smith,[7] and (2) the theory of probability integrals concerning thresholds as propounded by Crozier.[10] Crozier *et al.*[9] pointed out that with change in temperature the shift along the intensity axis predicted from the photochemical theory is in the direction opposite to what actually occurs.

Jahn[8] has proposed a new photochemical theory in which the Hecht's theory has been so modified as to fit the experimental data by Crozier and others. The theory has the following expression:

$$F=\frac{k_4 p}{C'}\left[k_1 I\ (a-x)\right]^q (F_{max}-F)^r \qquad (1)$$

In this formula F stands for CFF, F_{max} denotes CFF for sufficiently high intensities, I intensity, a the total available concentration of photosensitive substance, x the amount of photoproducts, 1/p the fraction of the time during which the light is on, k_4 the velocity constant of the secondary reaction which is dependent on temperature, and the other symbols C′, k_1, q and r represent constants independent of temperature. The factor $(F_{max}-F)^r$ is an arbitrary function without any photochemical ground. It has been added merely because it was necessary to make the theory agreeable to the experimental data by Crozier *et al.*[9] concerning the temperature dependence of the CFF—log intensity relation. As to this function f(F), Jahn says: "This function does not pertain to the photomechanism by which the sense-cell is stimulated but to the mechanism whereby the sensation of flicker approaches a maximum when the frequency of repetitive equivalent stimuli is increased. The controlling process which governs this phenomenon may lie any place in the catenary series of events between the reaction denoted by Eq (4) (the secondary reaction) and the sensation of flicker. Since this mechanism is undetermined, we may consider f(F) to be any function which will fit the data." The parenthesis in the quotation was given by the present authors.

The Jahn's theory indicates that the flicker fusion in which the central mechanism is involved cannot be explained by a purely photochemical theory. However, the flicker fusion in which no central process takes part, e.g. the flicker fusion of the slow potential in question may be accounted for on the purely photochemical basis. We attempted, therefore, to test the validity of the photochemical concept by Jahn for our data, and found that it was useful to explain the effect of temperature on the CFF—log intensity relation of the slow potential. For the reasons stated above the arbitrary factor $(F_{max}-F)^r$ was omitted from the Jahn's theory,

and the purely photochemical part, that is,

$$F=\frac{k_4 p}{C'}\left[k_1 I\ (a-x)\right]^q \qquad (2)$$

was applied to the data.

As the velocity constant of the secondary reaction k_4 increases with increasing temperatures, it may be derived from formula (2) that the maximal CFF becomes higher as the temperature is raised. This relation predicted from the theory conforms well to our data, as has been shown above.

Summary

A KCl-filled microelectrode was inserted into the retina of the carp to record an intracellular slow potential. Especially flicker and fusion of this potential in response to intermittent light was studied with special reference to temperature. The light-dark ratio of the stimulus was 1:1.

1. The critical fusion frequency (CFF) was different according to the types of elements. In the N-element which showed a simple negative-going response to a steady illumination, the CFF was lower than that of the A-element which showed a positive overswing at cessation of illumination. For example, the CFF of a N-element was 48 cps. at 29°C, while that of an A-element was 72 cps. at the same temperature.

2. The CFF—log intensity relation was investigated at three different temperatures. The general trend of the relation was very similar to those obtained by the psychophysical and behavorial methods, and a single smooth curve was sufficient to represent the data at each temperature.

3. The maximal CFF was found higher with increasing temperatures, and this feature and the general trend of the CFF—log intensity curve could be accounted for on the basis of the photochemical theory as formulated by Jahn.

References

1) Svaetichin, G., Acta physiol. Scand., 1953, **29**, Suppl. 106, 565.
2) Svaetichin, G., *ibid.*, 1956, **39**, Suppl. 134, 47.
3) Granit, R., Receptor and sensory perception. Yale Univ. Press, 1955.
4) Motokawa, K., Oikawa, T. & Tasaki, K., J. Neurophysiol., 1957, **20**, 186.
5) Tomita, T., Jap. J. Physiol., 1957, **7**, 80.
6) Oikawa, T., Ogawa, T. & Motokawa, K., J. Neurophysiol., in press.
7) Hecht, S., Shlaer, S. & Smith, E., Cold Spring Harbor Symposium, 1935, **3**, 237.
8) Jahn, T. L., J. Opt. Soc. Amer., 1946, **36**, 76.
9) Crozier, W. J., Wolf, E. & Zerrahn-Wolf, G., J. Gen. Physiol., 1936, **20**, 416.
10) Crozier, W. J., Proc. Nat. Acad. Sci., 1937, **23**, 71.

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Effect of Mental Work on Values of Electric Flicker

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Introduction

The problem of fatigue is of practical importance in our life. A number of methods or tests have hitherto been proposed for the measurement of fatigue. But, in practice, it is demanded to resort to a few methods by which the most fundamental features of fatigue can be tested. The procedure of measurement should be as simple as possible, and the result should be clear-cut and in good harmony with our physiological and psychological common sense. Motokawa and coworkers[1] have previously reported that these demands seemed, to some extent, to be satisfied with Motokawa's electric flicker method (EF), and that a flicker sensation caused by electric stimuluation of the eye could be used as a very convenient indicator for the measurement of fatigue. Moreover, Suzuki, Tukahara and co-workers[2),3),4),5)] investigated the effect of muscular labor and exercise on values of electric flicker (EFV). And then, many investigations were carried out by this Motokawa's method of EF. Tukahara[4)] suggested that the method might be an effective one for the measurement of fatigue caused by mental work, but mental fatigue has not yet been systematically studied with this method. Markkanen[1)] investigated the influence of examinations upon the count of eosinophile cells, and reported that the average count of the eosinophile cells decreased during the examination in all the students tested.

In the present experiment, the effect of mental work upon EFVs was systematically investigated in comparison with the effect of exercise upon them.

Experimental

Method

The subjects in the present experiment were 3 healthy males. For

30 minutes before each experiment, the subjects were laid down quietly on the bed. The measurements of EFVs were carried out for 10 minutes after the rest to obtain control values. The subjects performed exercise or mental work after the measurement of the control values. Similar measurements were made continuously for 60 minutes after exercise or mental work. But, in order to prevent fatigue which would be caused by too frequent measurements, a pause of 5 minutes was made from time to time during measurements in recovery.

The apparatus for electric flicker used in the present experiment was the same as described in Motokawa's report.[7] The stimulating current was increased and decreased automatically, to determine the threshold for appearance of flicker and that for its disappearance. The difference of both thresholds, S_1-S_2, was denoted by EFV. The EFV was expressed in terms of μA.

Results

The results obtained from 3 subjects who performed mental work (statistical calculation) for 60 minutes are represented in Fig. 1 (A, B and C). As can be seen in the 3 curves of Fig. 1, the EFV was found much

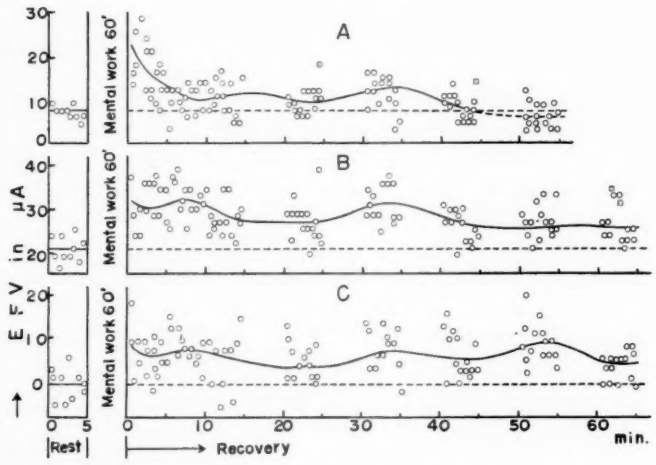

Fig. 1. Changes of electric flicker value (EFV) caused by mental work (calculation of statistics). A, B and C refer to different subjects.

higher immediately after the mental work than the control value. Recovery was very slow and remarkably prolonged. The EFV of one subject (A) recovered in about 45 minutes after mental work, but no complete

recovery was yet seen 60 minutes after mental work, in the other two subjects (B and C). That is to say, the difference between the EFV at this moment and control value before mental work was shown to be statistically significant. Further control experiments were carried out in which no mental work was imposed upon the subjects, the other experimental conditions being the same as above. The results obtained are shown in Fig. 2 (A and B). No such changes as in Fig. 1 can be seen in these control experiments. From this fact it is apparent that the changes of EFVs shown in Fig. 1 were caused by mental work.

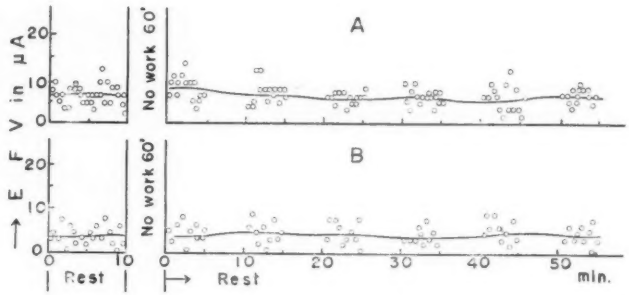

Fig. 2. Control experiments for those shown in Fig. 1. A and B refer to different subjects.

In the following experiments, the effect of exercise (running and steptests) was studied for comparison in the same subjects. The 2 subjects run for 30 minutes at a rate of 300 m. per minute. The results obtained are illustrated in Fig. 3 (A) and Fig. 4 (B). As can be seen in these figures, the EFV increased remarkably immediately after the running, but recovery was very prompt; the EFV almost returned to the initial value in 10–15 minutes. The difference between the EFVs obtained before and after the running became statistically insignificant in 20–30 minutes.

Next, the steptest was performed for 60 minutes by another subject (C). The steptest consisted of going up and down 2 stairs, 25 cm. high each, at a constant rate (12 times per minute). Before and after this test measurements of EFV were done as above. The result obtained was found very similar to those mentioned above (see Fig. 4 (C)).

In order to compare the characteristics of recovery curves for mental work and exercise, the six curves illustrated in Fig. 1, 3 and 4 are summalized in Fig. 5, in which increases above each control value are plotted as ordinates against recovery time in min. The curves concerning mental work are represented by thin lines, and those concerning exercise by thick lines. As can be seen in this figure, the value obtained immediately after

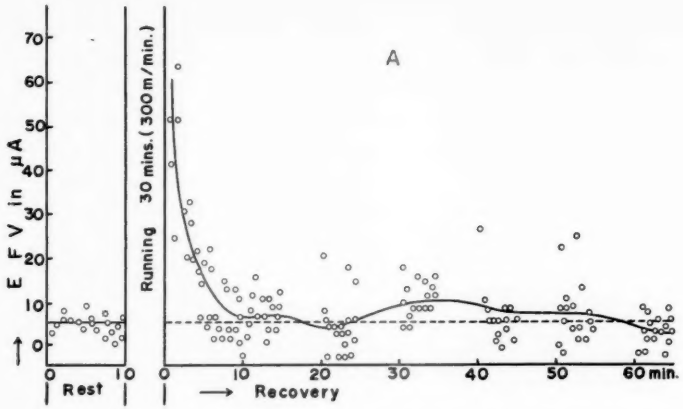

Fig. 3. Changes of EFV caused by exercise (running).

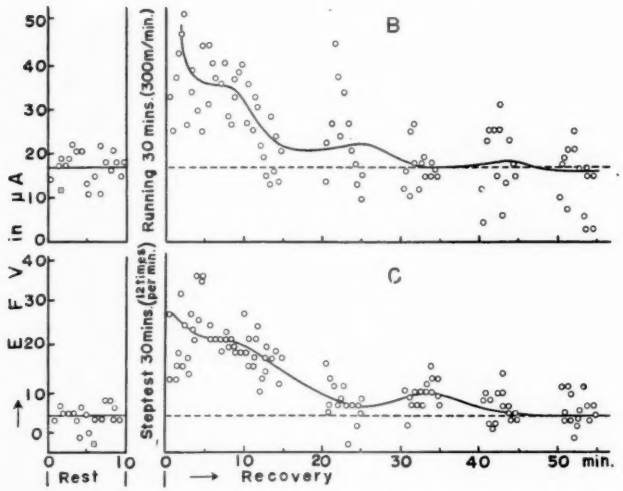

Fig. 4. Changes of EFV caused by exercise (running and steptest). B: Running. C: Steptest.

the exercise were remarkably higher than that after the mental work, but recovery was very prompt.

In the following experiment, the effect of light gymnastics performed for 5 minutes after the mental work was investigated. The results obtained are shown in Fig. 6. The EFVs obtained immediately after the gymnastics are of the same order as those obtained after the mental work alone, but

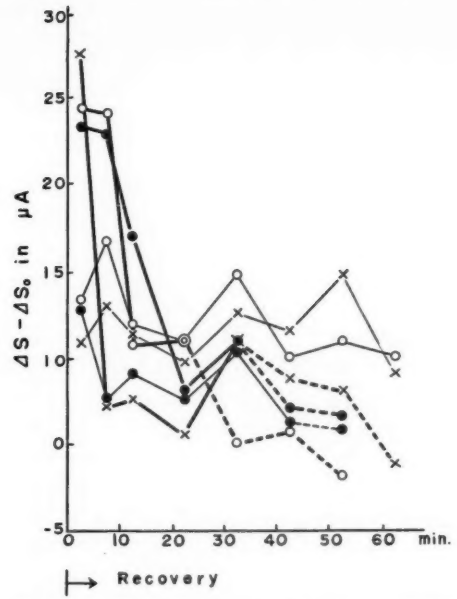

Fig. 5. Changes of EFVs caused by mental work and exercise. ΔS-ΔS_0: Increase of EFV above control value. Thick lines: Exercise. Thin lines: Mental work. Broken lines: Parts without significant difference from control value.

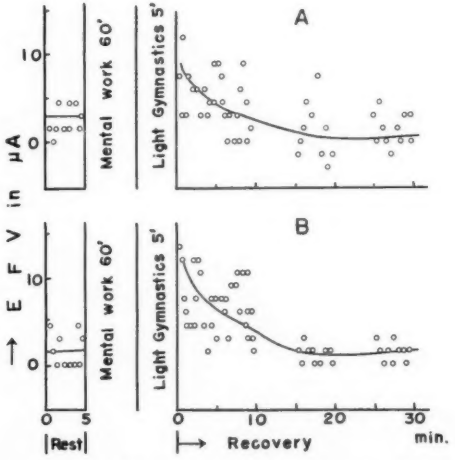

Fig. 6. Effects of light gymnastics performed for 5 minutes after mental work. A and B refer to different subjects.

recovery was not so prolonged ; 10 minutes after the gymnastics, no statistically significant difference from the control could be found. From what has been shown above, it is almost certain that, light gymnastics facilitate recovery from fatigue caused by mental work.

Discussion

A great number of methods or tests have hitherto been proposed for the measurement of fatigue. Matsushima[5] pointed out that the oxygen consumption or oxygen debt in writing, reading or talking (etc.) is so small that it can not be an indicator of fatigue caused by light work or mental work.

Certainly noteworthy is the observation by Markkanen[6] that the average counts of the eosinophile cells decreased appreciably during the examination in 112 students. But as he stated, the number of eosinophile cells shows remarkable individual variations, sometime spontaneous fluctations. Therefore it does not seem easy to measure fatigue caused by mental work by this or any other methods. We dare not to say that mental fatigue may be measured by the present method, but it seems that some characteristic features of mental fatigue may be revealed by this method, especially when compared with fatigue caused by muscular labor. The increase in EFV is only slight with mental fatigue, but recovery is remarkably prolonged as compared with recovery after physical work, so far as the EFV is concerned.

Orii[9] measured the fatigue caused by calculation and that by gymnastics with the method of flicker fusion frequency (FFF), and reported that recovery of FFF after the calculation was prolonged in comparison with that after the gymnastics. This observation is in line with our experiments.

Simonson[10] found a decrease of 5.2–6.6 flashes after calculation (blind-folded) and said that FFF might be used as an indicator of fatigue caused by mental work. The method of FFF seems to have something common with the present method.

Summay

By means of Motokawa's method of electric flicker (EF) we measured changes of electric flicker value (EFV) of 3 subjects caused by mental work (calculation of statistics) and exercise (running and steptests).

1. Immediately after mental work, the EFV increased. Recovery was very slow and much prolonged ; the difference between the EFVs measured before and 60 minutes after mental work was found statistically significant.

2. Immediately after exercise the EFV increased far more remarkably

than after mental work, but recovery was very prompt ; the difference of EFV from the control value was no longer significant after 20–30 min.

3. Effects of light gymnastics performed after mental work were investigated. Recovery from fatigue caused by mental work was facilitated by light gymnastics.

The authors have pleasure in acknowledging their indebtedness to Prof. K. Motokawa who has guided them kindly.

References

1) Motokawa, K. & Suzuki, K., Jap. Med. J., 1949, **1**, 200.
2) Suzuki, K., Tohoku J. Exp. Med., 1950, **52**, 17.
3) Suzuki, K., *ibid.*, 1950, **52**, 1.
4) Tukahara, S., Ebe, M. & Isobe, K., Tohoku Igaku Zassi (Jap.), 1950, **41**, 28.
5) Suzuki, K., Tukahara, S., Kobayashi, M. & Umetsu, J., *ibid.*, 1955, **49**, 313.
6) Markkanen, A., Acta physiol. Scand., 1955, **35**, 225.
7) Motokawa, K., Umetsu, J., Kobayashi, M. & Kameyama, M., Tohoku J. Exp. Med., 1956, **64**, 151.
8) Matsushima, S., J. of Science of Labour (Jap.), 1931, **2**, 641.
9) Orii, S., Hokkaido J. of Med. Science (Jap.), 1955, **30**, 349.
10) Simonson, E. & Brozek, J., Physiol. Rev., 1952, **32**, 349.

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Effect of Atropine on the Responsiveness of the Adrenal Medulla to Acetylcholine

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Feldberg, Minz and Tsudzimura[1] found that acetylcholine was released as a chemical transmitter from the endings of the adrenaline-secretory fibers. In fact, a marked increase in the adrenaline secretion rate is producible by an intravenous injection of acetylcholine. The administration of acetylcholine results in a remarkable fall in blood pressure. It brings an elevation of blood pressure when a small dose of atropine such as 1 mg. per kg. was given beforehand. However, that such a small dose of atropine as above does not affect the secretory function of the adrenal medulla is well established.

As concerns large doses of atropine, Kuré and his co-workers[2] observed that the augmentation of the adrenaline secretion of the adrenals causable by splanchnic nerves stimulation was wholly abolished by atropine. Contrariwise, Sato, Muto and Hatano[3] noted that the augmented adrenaline secretion after the application of morphine and insulin, both of which were proved to act through the central mechanism, was not affected by large doses of atropine.

Recently it was established that large doses of atropine was capable of blocking the ganglionic synapse.[4] The neural mechanism of excitation of the adrenal medulla is cholinergic and analogous to the ganglionic synapse. We have therefore undertaken to investigate quantitatively whether the adrenaline-secretory action of acetylcholine is inhibited by large doses of atropine.

Experimental

Methods

In 6 dogs, anesthetized with Evipan-sodium, the experiments were performed. The adrenal venous blood specimens were collected by the

lumbar route method.[5] For recording the blood pressure, a mercury manometer was connected with the femoral artery. The adrenaline content in the sampled adrenal venous blood was estimated colorimetrically by the method of Bloor and Bullen,[6] adrenaline (Sankyo Co.) being used as a standard.

Atropin. sulfur. (Boehringer), dissolved in 0.9% saline solution, was injected intravenously. In Exps. 1. & 2., acetylcholine in a dose of 2 mg. per kg. was injected intravenously. Acetylcholine in doses of 1.5 mg. and 1 mg. per kg. was injected in Exps. 3. & 4. and Exps. 5. & 6., respectively.

TABLE I

Effect of Atropine on the Augmented Adrenaline Secretion Causable by Acetylcholine

No. of experiment	Body weight and sex	No. of specimen	Duration of collection (sec.)	Quantity of blood (cc.)	Blood flow per kg. per minute (cc.)	Adrenaline in 1 cc. blood (γg.)	Adrenaline secretion rate (γg.) per kg. per minute	Body temp. (°C)	Room temp. (°C)
1	13.1 kg. ♀	1	30	1.7	0.26	0.14	0.04	39.1	20
		2	30	2.0	0.31	0.13	0.04		
				Acetylcholine (2 mg. per kg.)					
		3	60	0.7	0.05	12.4	0.62		
		4	60	4.0	0.31	0.22	0.07		
				Atropine (1 mg. per kg.)					
		5	30	1.4	0.21	0.13	0.03	39.8	19.5
				Acetylcholine (2 mg. per kg.)					
		6	60	2.6	0.20	3.6	0.72		
		7	60	1.9	0.15	0.20	0.03		
				Atropine (10 mg. per kg.)					
		8	60	2.5	0.19	0.06	0.01	39.8	19.5
				Acetylcholine (2 mg. per kg.)					
		9	60	2.0	0.15	4.3	0.65		
		10	60	1.9	0.15	0.19	0.03		
2	13.9 kg. ♂	1	30	1.8	0.26	*		36.8	20
		2	30	1.7	0.24	*			
				Acetylcholine (2 mg. per kg.)					
		3	60	1.6	0.12	6.2	0.74		
		4	60	2.4	0.17	0.43	0.07		
				Atropine (1 mg. per kg.)					
		5	30	1.4	0.20	*		36.8	20
				Acetylcholine (2 mg. per kg.)					
		6	60	4.6	0.33	5.9	1.9		
		7	60	4.9	0.35	0.09	0·03		
				Atropine (10 mg. per kg.)					
		8	60	2.2	0.16	*		37.1	20
				Acetylcholine (2 mg. per kg.)					
		9	60	2.6	0.19	4.0	0.76		
		10	60	2.8	0.20	*			

TABLE I—*concluded*

No. of experiment	Body weight and sex	No. of specimen	Duration of collection (sec.)	Quantity of blood (cc.)	Blood flow per kg. per minute (cc.)	Adrenaline in 1 cc. blood (γg.)	Adrenaline secretion rate (γg.) per kg. per minute	Body temp. (°C)	Room temp. (°C)
3	10.0 kg. ♂			Atropine (1 mg. per kg.)					
		1	30	2.0	0.40	0.06	0.02	36.9	21
				Acetylcholine (1.5 mg. per kg.)					
		2	60	2.8	0.28	1.4	0.39		
		3	60	2.5	0.25	*			
				Atropine (10 mg. per kg.)					
		4	30	1.3	0.26	0.09	0.02	36.9	21
				Acetylcholine (1.5 mg. per kg.)					
		5	60	3.8	0.38	1.2	0.46		
		6	60	4.2	0.42	*			
4	14.6 kg. ♂			Atropine (1 mg. per kg.)					
		1	30	4.3	0.59	0.06	0.04	37.1	21.5
				Acetylcholine (1.5 mg. per kg.)					
		2	60	7.9	0.54	2.7	1.5		
		3	60	7.1	0.49	*			
				Atropine (10 mg. per kg.)					
		4	30	3.0	0.41	*		36.9	21
				Acetylcholine (1.5 mg. per kg.)					
		5	60	7.0	0.48	3.6	1.7		
		6	60	7.0	0.48	0.06	0.03		
5	11.4 kg. ♀			Atropine (1 mg. per kg.)					
		1	60.	2.1	0.19	0.12	0.02	36.1	22
				Acetylcholine (1 mg. per kg.)					
		2	60	2.6	0.23	0.58	0.13		
		3	60	1.9	0.17	0.14	0.02		
				Atropine (10 mg. per kg.)					
		4	90	1.0	0.06	0.09	0.005	36.1	22
				Acetylcholine (1 mg. per kg.)					
		5	60	1.6	0.14	0.79	0.11		
		6	60	1.0	0.09	0.18	0.02		
6	10.0 kg. ♀			Atropine (1 mg. per kg.)					
		1	30	1.9	0.38	0.08	0.03	39.0	27
				Acetylcholine (1 mg. per kg.)					
		2	60	3.9	0.39	2.3	0.90		
		3	60	4.1	0.41	0.07	0.03		
				Atropine (10 mg. per kg.)					
		4	30	1.4	0.28	*		39.5	27
				Acetylcholine (1 mg. per kg.)					
		5	60	4.1	0.41	2.1	0.86		
		6	60	3.8	0.38	*			

* Less than 0.05 γg.

The duration of intravenous injection of acetylcholine was in all cases 15 seconds.

Results

The results are shown in Table I.

In Exp. 1, the rate of adrenaline secretion before the first acetylcholine injection was 0.04 μg. per kg. of body weight per minute. By the administration of acetylcholine in a dose of 2 mg. per kg. the rate was increased up to 0.62 μg., which was estimated in the first 60-second period after the start of injection. In the second 60-second period it was 0.07 μg. Eighty minutes after the first acetylcholine administration, 1 mg. atropine per kg. was injected. Then, acetylcholine in the same dose as before was injected again. The rate of adrenaline secretion before the second acetylcholine injection was 0.03 μg. On receiving acetylcholine, the rate of adrenaline secretion was increased up to 0.72 μg. during the first 60-second period. In the second 60-second period it was estimated as 0.03 μg. About 20 minutes later, 10 mg. atropine per kg. was given. And about 15 minutes after atropine, the injection of acetylcholine in the same dose as before was made. The rate of adrenaline secretion before acetylcholine was 0.01 μg. By the injection of acetylcholine it was augmented and determined as 0.65 μg. in the first 60-second period. In the second 60-second period it was 0.03 μg.

In Exp. 2, the rate of adrenaline secretion before the first acetylcholine injection was so small that it was immeasurable and it increased up to 0.74 μg. per kg. per minute at the first 60-second period after the injection of acetylcholine in a dose of 2 mg. per kg. About half an hour later the application of atropine in a dose of 1 mg. per kg. was made and then acetylcholine in the same dose as before was injected again. The rate of adrenaline secretion before the acetylcholine injection was immeaurably small. After the acetylcholine injection it increased 1.9 μg. in the first 60-second period. About 25 minutes later, 10 mg. atropine per kg. was given. And after that the third acetylcholine injection was made. The secretion rate before acetylcholine was immeasurably small. After the injection of acetylcholine, it was found to have increased and was estimated as 0.76 μg. in the first 60-second period.

In Exp. 3, the injection of acetylcholine in a dose of 1.5 mg. per kg. was performed after the application of atropine in a dose of 1 mg. per kg. The rate of adrenaline secretion before acetylcholine was 0.02 μg. per kg. per minute. By the injection of acetylcholine the secretion rate was increased, the highest rate being 0.39 μg., which was measured in the first 60-second period after the start of injection. About 40 minutes later, atropine in a dose of 10 mg. per kg. was given and then the injection of acetylcholine in the same dose as before was made again. The secretion rate before the second acetylcholine was estimated as 0.02 μg. After

acetylcholine it increased remarkably, 0.46 μg. being reached in the first 60-second period.

Similar results were obtained in Exp. 4.

After the application of atropine in a dose of 1 mg. per kg., acetylcholine in a dose of 1.5 mg. per kg. was injected. By the acetylcholine injection, the adrenaline secretion rate was accelerated enormously. It increased from 0.04 μg. per kg. per minute to 1.5 μg. in the first 60-second period after the start of injection. About 25 minutes after the first acetylcholine injection, atropine in a dose of 10 mg. per kg. was given and after a while the second acetylcholine injection was made. The adrenaline secretion rate before the second acetylcholine injection was too small to be estimated. On injecting acetylcholine it increased up to 1.7 μg. during the first 60-second period.

In Exp. 5, 1 mg. acetylcholine per kg. was injected after the administration of 1 mg. atropine per kg. The rate of adrenaline secretion before acetylcholine was 0.02 μg. per kg. per minute. After the injection of acetylcholine it was found to have increased up to 0.13 μg. in the first 60-second period. After about 10 minutes, 10 mg. atropine per kg. was given and then acetylcholine in the same amount as before was injected. The secretion rate before the second acetylcholine was 0.005 μg. On injecting acetylcholine, a definite increase in the secretion rate was observed and was determined as 0.11 μg. in the first 60-second period.

Almost the same results were obtained in Exp. 6.

After administration of atropine in a dose of 1 mg. per kg., acetylcholine in an amount of 1 mg. per kg. was injected. Before acetylcholine the adrenaline secretion rate was 0.03 μg. per kg. per minute. By the injection of acetylcholine it was accelerated, 0.90 μg. being estimated in the first 60-second period. Half an hour later, the injection of acetylcholine in the same dose as before was made after the application of atropine in a dose of 10 mg. per kg. The rate of secretion before acetylcholine was too small to be estimated. After acetylcholine, a remarkable increase in the adrenaline secretion was observed, 0.86 μg. being determined in the first 60-second period.

Summary

The experiments were performed in dogs anesthetized with Evipan-sodium. The adrenal venous blood was collected through the lumbar route and was estimated for adrenaline using the arseno-molybdic acid method of Bloor and Bullen. Intravenous injection of acetylcholine in doses of 2.0 mg., 1.5 mg. or 1.0 mg. per kg. of body weight was made after administrations of atropine in doses of 1 mg. and 10 mg. per kg., respectively. In some cases it was also made without administering atropine previously.

After the administration of atropine in a dose of 10 mg. per kg., a definite increase in the adrenaline secretion rate was elicited by the injection of acetylcholine in almost the same magnitude as that caused by acetylcholine after atropine in a dose of 1 mg. per kg. or without atropine.

From these experimental results, it may be concluded that the adrenaline-secretory action of acetylcholine is not inhibited even by large doses of atropine.

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References

1) Feldberg, W., Minz, B. & Tsudzimura, H., J. Physiol., 1934, **81**, 286.
2) Kuré, K., Wada, Y. & Okinaka, S., Quart. J. Exp. Physiol., 1932, **21**, 227.
3) Sato, H., Muto, T. & Hatano, M., Tohoku J. Exp. Med., 1938, **34**, 393.
4) Dutta, N. K., Brit. J. Pharmacol., 1949, **4**, 197.
5) Satake, Y., Sugawara, T. & Watanabe, M., Tohoku J. Exp. Med., 1927, **8**, 501.
6) Bloor, W. K. & Bullen, S. S., J. Biol. Chem., 1941, **138**, 727.

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Effect of Diisopropylfluorophosphate on the Secretory Function of the Adrenal Medulla

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Considerable evidence has accumulated to show that diisopropylfluorophosphate (DFP) is capable of affecting the cholinergic mechanism as an inhibitor of cholinesterase. Since the neural mechanism of the adrenal medullary secretion is cholinergic,[1] it is probable that DFP has some effects on the adrenaline-secretory function of the adrenal gland. However, no clear-cut evidence has been provided in this respect.

Thus, the present investigation was undertaken to know how DFP acts on the adrenaline secretion of the adrenal gland and on the adrenaline-secretory effects of acetylcholine and carbaminoylcholine.

Experimental

Methods

Fourteen dogs, 7.3–14.4 kg. in weight, were used. The experiments were performed under Evipan-sodium anesthesia.

In order to collect the adrenal venous blood, the lumbar route method of Satake *et al.*[2] was employed. The adrenaline content of the sampled adrenal venous blood was estimated chemically by means of the Bloor and Bullen's arseno-molybdic acid method,[3] adrenaline base (Sankyo Co.) being used as the reference standard. The femoral artery was connected with the mercury manometer to register the blood pressure.

In the first part of the present investigation, experiments were carried out to know how DFP itself affects the adrenaline secretion rate of the adrenal gland. After control adrenal venous blood specimens were collected, DFP (Sumitomo Co.) was injected intravenously in a dose of 1.0 mg. per kg. of body weight in 60 seconds. During the first 60-second period after the start of injection and at 5, 10, 20, 30, 45 and 60 minutes after DFP, the adrenal venous blood was collected.

Three sets of experiments were performed.

Subsequently, some experiments were made to elucidate the effect of DFP on the adrenaline-secretory action of acetylcholine. Acetyl-

choline chloride (Hoffmann La Roche) in a dose of 1.0 mg. per kg. was injected intravenously in 15 seconds. After a while DFP in doses of 0.3–1.0 mg. per kg. was administered intravenously and then acetylcholine in the same dose as before was injected again. The adrenal venous blood was collected before injection of acetylcholine and successively during the first and the second 60-second period after the start of acetylcholine injection.

In the last step, carbaminoylcholine chloride (Doryl, Merck) in a dose of 0.4 mg. per kg. was injected instead of acetylcholine. DFP was applied intravenously in a dose of 1.0 mg. per kg.

In these experiments, atropine in a dose of 1.0 mg. per kg. was injected some minutes before injections of acetylcholine and carbaminoylcholine in order to suppress intense depressor actions of choline esters.

Results

(*A*) *Effect of DFP on the adrenaline secretion of the adrenal gland*

The results obtained in 3 dogs are summarized in Table I.

In Dog 1, the adrenaline secretion rate before DFP injection was 0.02–0.03 μg. per kg. per minute. After DFP, there was not any definite increase in the secretion rate. It was estimated as 0.03 μg. in the first 60-second period after the start of injection and at 60 minutes after DFP. At 5, 10, 20, 30 and 45 minutes after DFP, it was measured as 0.04 μg.

TABLE I

Adrenaline Secretion Rate of Adrenal Gland after Injection of DFP

No. of animal	Body weight and sex	Dose of DFP per kg. (mg.)	Adrenaline secretion rate per kg. per minute (μg.)							
			Before injection	After injection (min.)						
				0–1	5–6	10–11	20–21	30–31	45–46	60–61
1	9.0 kg. ♂	1.0	0.02–0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.03
2	10.8 kg. ♀	1.0	0.04	0.03	0.06	0.05	0.03	0.04	0.02	0.02
3	9.6 kg. ♂	1.0	*	*	0.02	*	*	*	*	*

* immeasurably small.

Almost the same results were obtained in Dog 2. Before DFP, the rate of adrenaline secretion was 0.04 μg. per kg. per minute. After DFP, no increase in the rate of secretion was found. It was estimated as 0.03 μg. in the first 60-second period. And at 5, 10, 20, 30, 45 and 60 minutes after DFP it was 0.06 μg., 0.05 μg., 0.03 μg., 0.04 μg., 0.02 μg. and 0.02 μg., respectively.

In Dog 3, the adrenaline secretion rate before DFP was too small to

be estimated. It was immeasurably small just after the start of DFP injection and at 10, 20, 30, 45 and 60 minutes after DFP. At 5 minutes after DFP it was measured as 0.02 μg.

(*B*) *Effect of DFP on the adrenaline-secretory action of acetylcholine*

The results are set out in Table II.

DFP in a dose of 0.3 mg. per kg. was applied in Dog 4. The basal rate of adrenaline secretion was 0.03–0.04 μg. On receiving acetylcholine, the secretion rate was increased during the first 60-second period after the start of injection, 0.29 μg. being measured. And then it resumed the

TABLE II

Effect of DFP on the Augmented Adrenaline Secretion Causable by Acetylcholine

No. of animal	Body weight and sex	Dose of DFP per kg. (mg.)	Adrenaline secretion rate per kg. per minute (μg.)					
			Before DFP			After DFP		
			Before acetylcholin	After acetylcholine (sec.)		Before acetyl-choline	After acetylcholine (sec.)	
				0–60	60–120		0–60	60–120
4	7.3 kg. ♂	0.3	0.03-0.04	0.29	0.03	0.02	0.53	0.03
5	12.2 kg. ♂	0.4	0.02	0.30	0.04	0.02	0.70	0.05
6	9.5 kg. ♂	0.6	0.03	0.09	0.03	0.03	0.70	*
7	14.4 kg. ♂	0.6	0.01	0.22	0.02	0.01	0.69	*
8	14.1 kg. ♂	1.0	0.01-0.02	0.17	*	*	1.5	0.03
9	8.0 kg. ♀	1.0	0.01-0.02	0.47	0.19	0.01	1.4	0.02

* immeasurably small.

initial value in the second 60-second period. After a while, an intravenous injection of DFP was made, and then the second acetylcholine injection was performed. The adrenaline secretion rate before the second acetylcholine injection was 0.02 μg. After the injection, a marked increase in the adrenaline secretion rate was found and it was estimated as 0.53 μg. in the first 60-second period. In the second 60-second period it was 0.03 μg.

In Dog 5, DFP was applied in a dose of 0.4 mg. per kg. After the first acetylcholine injection the rate of adrenaline secretion increased from 0.02 μg. up to 0.30 μg. per kg. per minute in the first 60-second period after the start of injection. In the next 60-second period, it resumed the initial rate. On receiving acetylcholine after DFP, the rate of adrenaline secretion was markedly accelerated. It increased from 0.02 μg. and reached 0.70 μg. in the first 60-second period. In the following 60-second period, it was 0.05 μg.

DFP in a dose of 0.6 mg. per kg. was applied in Dogs 6 and 7.

In Dog 6, the initial rate of adrenaline secretion was 0.03 μg. per kg. per minute. On receiving acetylcholine there was a slight but definite increase in the secretion rate. It was estimated as 0.09 μg. in the first 60-second period. By the injection of acetylcholine after DFP, a remarkable increase in the adrenaline secretion rate was elicited. It was found to be 0.70 μg. in the first 60-second period, the pre-injection rate being 0.03 μg.

In Dog 7, the adrenaline secretion rate before the first acetylcholine injection was 0.01 μg. per kg. per minute. It was measured as 0.22 μg. and 0.02 μg. during the first and the second 60-second period after the first acetylcholine injection, respectively. After DFP, the rate of secretion before the second acetylcholine injection was 0.01 μg. It was accelerated markedly by the injection of acetylcholine and reached 0.69 μg. in the first 60-second period. In the second 60-second period it was immeasurably small.

DFP in a dose of 1.0 mg. per kg. was applied in Dogs 8 and 9.

In Dog 8 the secretion rate before the first acetylcholine injection was 0.01–0.02 μg. per kg. per minute. After the injection it increased up to 0.17 μg. in the first 60-second period. After DFP the intravenous injection of acetylcholine caused a marked increase in the adrenaline secretion rate. It was measured as 1.5 μg. in the first 60-second period.

In Dog 9 the rate of adrenaline secretion before the first acetylcholine injection was 0.01–0.02 μg. per kg. per minute. By the injection of acetylcholine it was increased up to 0.47 μg. in the first 60-second period. In the following 60-second period, a somewhat increased rate of secretion was still found and it was 0.19 μg. After DFP the secretion rate before the second acetylcholine injection was 0.01 μg. On receiving acetylcholine it was increased remarkably, the maximal value being 1.4 μg., which was found in the first 60-second period.

(*C*) *Effect of DFP on the adrenaline-secretory action of carbaminoylcholine*

The results are epitomized in Table III.

In Dog 10, the rate of adrenaline secretion before the first carbaminoylcholine injection was 0.03–0.07 μg. per kg. per minute. After the injection, it was accelerated markedly, 1.4 μg. being reached in the first 60-second period after the start of injection. And then in the following 60-second period a slightly augmented rate was still observed and it was 0.13 μg. Twenty-two minutes later, an injection of DFP was made and then the second carbaminoylcholine injection was performed. The adrenaline secretion rate before carbaminoylcholine was immeasurably small. After injection, the secretion rate increased considerably, 1.3 μg. being measured in the first 60-second period. In the second 60-second period, it was 0.08 μg.

TABLE III

Effect of DFP on the Augmented Adrenaline Secretion Causable by Carbaminoylcholine

No. of animal	Body weight and sex	Dose of DFP per kg. (mg.)	Adrenaline secretion rate per kg. per minute (μg.)					
			Before DFP			After DFP		
			Before carbaminoylcholine	After carbaminoylcholine (sec.)		Before carbaminoylcholine	After carbaminoylcholine (sec.)	
				0–60	60–120		0–60	60–120
10	12.4 kg. ♀	1.0	0.03–0.07	1.4	0·13	*	1.3	0.08
11	8.6 kg. ♀	1.0	0.01	0.82	0.05	*	0.79	0.05
12	7.8 kg. ♀	1.0	0.05	0.10	0.05	0.04	0.14	0.03
13	8.6 kg. ♂	1.0	0.03	3.7	0.18	*	3.1	0.14
14	10.1 kg. ♀	1.0	*	0.25	0.75	0.05	0.26	0.54

* immeasurably small.

Almost the same results were obatained in Dog 11. The adrenaline secretion rate before the first carbaminoylcholine injection was too small to be estimated. On receiving carbaminoylcholine, it increased and reached 0.82 μg. per kg. per minute in the first 60-second period. In the next 60-second period it was 0.05 μg. An injection of carbaminoylcholine was made after application of DFP. The adrenaline secretion rate before the second carbaminoylcholine injection was immeasurably small. After the injection it was increased up to 0.79 μg. in the first 60-second period. In the second 60-second period it was measured as 0.05 μg.

In Dog 12, the adrenaline secretion rate before the first carbaminoylcholine injection was 0.05 μg. per kg. per minute. The carbaminoylcholine injection caused an increase in the secretion rate, 0.10 μg. being measured in the first 60-second period. After a while an application of DFP was made and then carbaminoylcholine was injected again. Before the second carbaminoylcholine injection the adrenaline secretion rate was 0.04 μg. On receiving carbaminoylcholine, the rate of secretion was increased and reached 0.14 μg. in the first 60-second period.

Similar results were obtained in Dog 13. The initial rate of adrenaline secretion was 0.03 μg. per kg. per minute. It was increased by the carbaminoylcholine injection up to 3.7 μg. in the first 60-second after the start of injection. In the following 60-second period it was measured as 0.18 μg. After DFP the secretion rate was increased by the second carbaminoylcholine injection from immeasurably small rate up to 3.1 μg. in the first 60-second period. And then in the second 60-second period it was measured as 0.14 μg.

In Dog 14, the rate of adrenaline secretion before the first carbaminoylcholine injection was too small to be quantified. On receiving carbaminoylcholine, a noticeable increase in the secretion rate such as 0.25 μg. and 0.75 μg. per kg. per minute was found in the first and the second 60-second period, respectively. After a while an injection of DFP was made, and then carbaminoylcholine was injected again. The adrenaline secretion rate before the second carbaminoylcholine injection was 0.05 μg. After the injection the rate of secretion was increased definitely and was measured as 0.26 μg. and 0.54 μg. in the first and the second 60-second period, respectively.

Discussion

The results mentioned above indicate that DFP, in the dose I have used, is not capable of increasing the adrenaline secretion rate in dogs. As a matter of course, it may be possible that the dose of DFP was too small to produce an increase in the rate of secretion.

The adrenaline secretion rate was increased by intravenous injection of acetylcholine in a dose of 1.0 mg. per kg. And this adrenaline-secretory action of acetylcholine was proved to be enhanced definitely by DFP. In small doses of DFP such as 0.3–0.4 mg. per kg., however, this potentiating effect was rather slight. In dogs 4 and 5, in which DFP was given in doses of 0.3–0.4 mg. per kg., the rate of adrenaline secretion augmented by acetylcholine before DFP was 0.3 μg. per kg. per minute and that after DFP was measured as 0.5 and 0.7 μg. respectively; *i.e.* the adrenaline secretion rate increased by acetylcholine after DFP was only about double as much as that before DFP. In large doses of DFP such as 1.0 mg., however, the potentiating effect of DFP was found to be more intense.

The marked potentiating effect of eserine upon the adrenaline-secretory action of acetylcholine has been well established. It was also confirmed in the recent investigation of Suzuki, Tanaka and Yamashita[4] in our laboratory. Eserine in a dose of 0.3 mg. per kg. was applied in their experiments. While acetylcholine in doses of 0.3–0.4 mg. per kg. caused before eserine no or only slight increase in the adrenaline secretion rate, the same dose of acetylcholine, when given after eserine, provoked a marked increase, the maximal rate being measured as 0.86–1.8 μg. per kg. per minute. Comparing these data with those of the present investigation, it is evident that the potentiating effect of eserine upon the adrenaline-secretory action of acetylcholine is several times as much intense as that of DFP.

Brown, Burns and Feldberg[5] demonstrated in their experiments that DFP, like eserine, enhanced the tension response of the tibialis anterior to single maximal motor nerve volley, and the dose of DFP necessary for

producing the potentiating was several times as large as that of eserine. They inferred that the difference between the potentiating actions of DFP and of eserine was due to a greater affinity of DFP for pseudo- rather than for true-cholinesterase. Their explanation seems to be applicable for the experimental results of the present investigation.

An intravenous injection of carbaminoylcholine induced a definite increase in the adrenaline secretion before and after DFP as well. Adrenaline-secretory action of carbaminoylcholine was proved not to be influenced by the pretreatment of DFP. Similar results were already obtained in the eserine experiments of Suzuki and his co-workers.[4] These are explicable with the fact that carbaminoylcholine is not hydrolysed by cholinesterase.

Summary

The adrenal venous blood was sampled by the lumbar route method from dogs anesthetized with Evipan-sodium and was analysed for adrenaline by the Bloor and Bullen's colorimetric method.

The adrenaline secretion rate was not altered by the intravenous injection of DFP in a dose of 1.0 mg. per kg.

On receiving acetylcholine in a dose of 1.0 mg. per kg., the adrenaline secretion rate was increased, and this action of acetylcholine was enhanced definitely by the pretreatment of DFP in doses of 0.3–1.0 mg. per kg.

An intravenous injection of carbaminoylcholine in a dose of 0.4 mg. per kg. induced a considerable increase in the adrenaline secretion rate. However, this action was found to be almost unaffected by the previous administration of DFP in a dose of 1.0 mg. per kg.

I am grateful to Prof. T. Suzuki and Dr. K. Yamashita for their interest and help.

References

1) Feldberg, W., Minz, B. & Tsudzimura, H., J. Physiol., 1934, **81**, 286.
2) Satake, Y., Sugawara, T. & Watanabe, M., Tohoku J. Exp. Med., 1927, **8**, 501.
3) Bloor, W. R. & Bullen, S. S., J. Biol. Chem., 1941, **138**, 727.
4) Suzuki, T., Tanaka, I. & Yamashita, K., Tohoku J. Exp. Med., 1958, **68**, 133.
5) Brown, G. L., Burns, B. D. & Feldberg W., J. Physiol., 1948, **107**, 346.

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Effect of Insulin Hypoglycemia upon the Noradrenaline and Adrenaline Content of the Adrenal Gland in Toads

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It has been reported by some authors that the adrenal gland of cold blooded animals such as frogs and toads contains considerable amount of noradrenaline together with adrenaline.

However, to our knowledge, few studies have been published on the noradrenaline and adrenaline content of the adrenals in such animal species under various conditions.

The present investigation was undertaken to know the effect of insulin hypoglycemia upon the noradrenaline and adrenaline content of the adrenal glands in toads.

Experimental

Methods

Male and female toads were used. The adrenal gland together with the kidney was extirpated and the renal tissue was cut off from the adrenal gland as completely as possible.

The adrenal gland was then extracted with 4% trichloracetic acid solution and estimated for noradrenaline and adrenaline by the use of the permanaganate method of Suzuki and Ozaki.[1] Synthetic L-adrenaline (Merck) and DL-noradrenaline (Sankyo) were used as the reference standard.

Results

(*1*) *The noradrenaline and adrenaline content of the adrenal gland in normal toads*

Ten toads were employed. The data are shown in Table I.

The noradrenaline content per gland was estimated as 0.018–0.042 mg. in the left gland and 0.021–0.045 mg. in the right gland, the mean values being 0.033 mg. and 0.035 mg., respectively. The adrenaline content per

gland was 0.032–0.080 mg. in the left gland and 0.033–0.080 mg. in the right gland, the mean values being 0.059 mg. in each of both glands.

Computed per kg. of body weight, it was 0.107 (0.078–0.143) mg. and 0.114 (0.090–0.145) mg. for noradrenaline, 0.187 (0.154–0.244) mg. and 0.187 (0.159–0.236) mg. for adrenaline in the left and the right gland, respectively.

The relative amount of noradrenaline in the average was 36 per cent and 37 per cent in the left and the right gland, respectively.

(*2*) *Effect of insulin hypoglycemia on the noradrenaline and adrenaline content of the adrenal gland in toads*

Ten toads were used in this series of experiments.

Insulin was administered subcutaneously in a dose of 15 units per kg. of body weight. After 10 hours the adrenal glands were extirpated and the noradrenaline and adrenaline content was measured.

The noradrenaline content per gland was estimated as 0.018–0.045 mg. in the left gland and 0.021–0.043 mg. in the right gland, the average values being 0.032 mg. and 0.033 mg., respectively. The adrenaline content per gland was 0.026–0.061 mg. in the left gland and 0.025–0.059 mg. in the right gland, respectively. The mean values were 0.042 mg. in both sides.

The amount of noradrenaline per kg. of body weight was 0.090 (0.072–0.104) mg. in the left and 0.091 (0.077–0.104) mg. in the right gland. The adrenaline content per kg. was 0.117 (0.097–0.139) mg. and 0.116 (0.100–0.134) mg. in the left and in the right adrenal gland, respectively.

The relative noradrenaline amount in the mean was 43 per cent in the left gland and 44 per cent in the right gland, respectively.

Thus it was elucidated that the adrenaline content of the adrenal per kg. of body weight in these insulin-treated toads was distinctly smaller than that in normal toads. The difference was statistically highly significant ($P<0.001$).

In contrast, the noradrenaline content of the adrenal per kg. of body weight in insulin-treated toads was only slightly smaller than that in normal toads.

(*3*) *Effect of section of the splanchnic nerves on the decrease in the adrenaline content of the adrenal gland producible by insulin hypoglycemia*

In the control experiments, the right splanchnic nerves were cut through the lumbar route in 10 toads and 6 days after splanchnicotomy the noradrenaline and adrenaline content of the adrenal gland was estimated. No application of insulin was made.

In these experiments, the noradrenaline content in the left gland with intact innervation was estimated as 0.027 (0.018–0.035) mg. and that in the right, splanchnicotomized gland as 0.028 (0.021–0.038) mg. The

adrenaline content per gland was 0.046 (0.035–0.063) mg. and 0.047 (0.035–0.058) mg. in the left and the right gland, respectively.

The amount of noradrenaline per kg. of body weight was calculated as 0.120 (0.075–0.150) mg. and 0.125 (0.081–0.150) mg. and the amount of adrenaline per kg. as 0.203 (0.125–0.242) mg. and 0.206 (0.150–0.248) mg. in the left and the right gland, respectively. The difference between estimates of the right splanchnicotomized gland and the left gland with intact innervation was small and statistically not significant ($P>0.1$).

The experiments proper were performed in 9 toads.

The splanchnic nerves were cut in the right side and after 6 days subcutaneous injection of insulin in a dose of 15 units per kg. was made. Ten hours after insulin injection the adrenal gland was extirpated and estimated for noradrenaline and adrenaline.

The noradrenaline content per gland was 0.032 (0.015–0.063) mg. in the left and 0.033 (0.018–0.069) mg. in the right adrenal gland. The adrenaline content per gland was 0.035 (0.014–0.062) mg. in the left gland and 0.054 (0.029–0.092) mg. in the right gland.

The noradrenaline content per kg. of body weight was calculated as 0.133 (0.065–0.203) mg. and 0.139 (0.078–0.223) mg. in the left and in the right gland, respectively. The adrenaline content per kg. was 0.142 (0.094–0.172) mg. in the left gland with intact innervation and 0.227 (0.171–0.297) mg. in the right, splanchnicotomized gland. The difference between adrenaline estimates was statistically highly significant ($P<0.001$).

There was highly significant difference between the adrenaline content of the left gland with intact innervation in insulin-treated toads and that of the left gland in non-treated control toads ($P<0.01$), whereas no difference was found between the adrenaline estimate of the right splanchnicotomized gland in insulin-treated toads and that in non-treated control toads ($P>0.1$). Thus the depletion of adrenaline content of the adrenal gland was found to be fully nullified by the section of the splanchnic nerves.

Discussion

In the first set of experiments the noradrenaline and adrenaline content of the adrenal gland in normal toads was estimated. Relative noradrenaline content was measured as 37 (29–46) %. In Argentine toads, Bufo arenarum Hensel, the relative noradrenaline content of the adrenal gland was estimated by the hands of Houssay, Gerschman and Rapela[2] as 45 (35–58) %. Rapela and Gorden[3] found in the same animal species that the adrenal gland contained both noradrenaline and adrenaline in almost the same amount in summer (from December to March). In other seasons, however, especially at the end of autumn (May) the adrenaline

TABLE I

Noradrenaline and Adrenaline Content of the Adrenal Gland in Toads

No. of animal Body weight and sex	Noradrenaline (mg.)				Adrenaline (mg.)				Per cent noradrenaline	
	Per gland		Per kg. of body weight		Per gland		Per kg. of body weight			
	L	R	L	R	L	R	L	R	L	R
1. 200 g. ♂	0.021	0.021	0.105	0.105	0.032	0.033	0.160	0.165	40	39
2. 220 g. ♀	0.018	0.022	0.082	0.100	0.034	0.035	0.154	0.159	35	38
3. 250 g. ♀	0.029	0.029	0.116	0.116	0.061	0.059	0.244	0.236	32	33
4. 260 g. ♂	0.037	0.038	0.143	0.145	0.064	0.060	0.238	0.230	37	39
5. 320 g. ♂	0.039	0.045	0.122	0.141	0.050	0.051	0.157	0.159	43	46
6. 400 g. ♀	0.036	0.041	0.090	0.102	0.069	0.071	0.173	0.178	34	36
7. 430 g. ♀	0.036	0.040	0.084	0.092	0.080	0.080	0.186	0.186	31	33
8. 350 g. ♂	0.042	0.041	0.120	0.117	0.065	0.067	0.185	0.191	39	38
9. 400 g. ♂	0.031	0.036	0.078	0.090	0.077	0.074	0.192	0.185	29	32
10. 300 g. ♀	0.040	0.039	0.133	0.130	0.056	0.056	0.187	0.187	41	41
Mean	*0.033*	*0.035*	*0.107*	*0.114*	*0.059*	*0.059*	*0.187*	*0.187*	*36*	*37*

content was found to be much smaller than that of noradrenaline, the relative noradrenaline content being not less than 50%.

The estimate of relative noradrenaline amount of the present writer is therefore somewhat smaller than those of the Argentine investigators.

In the second set of experiments the adrenaline content of the adrenal gland was proved to be markedly reduced by insulin hypoglycemia. However, no distinct alterations of noradrenaline content were evoked by insulin hypoglycemia.

Similar results were obtained in cats and rats by some investigators. In cats the adrenaline content of the adrenals was found by Hökfelt[4] to be highly reduced after insulin administration, though there was not any alteration in the noradrenaline content.

In rats Burn, Hutcheon and Parker[5] demonstrated that the application of insulin resulted in a depletion of adrenaline in the adrenal gland, the maximal depletion being observed at 8 hours after insulin. There was, however, an increase in the relative noradrenaline content for several

hours after insulin. Similar results were obtained in rats by Outschoorn[6]. The increase in the noradrenaline content of the adrenal gland in early period after insulin was small in the magnitude and seemed to be non-significant. At 12–16 hours after insulin there was a marked depletion of adrenaline in the adrenal gland accompanied with a slight diminution of noradrenaline content.

Since in the present investigation the noradrenaline and adrenaline contents were estimated only at 10 hours after insulin, elucidation in toad adrenals of the time relations awaits further investigation.

The decrease in adrenaline of the adrenal gland producible by insulin hypoglycemia may be explicable by either of two mechanisms; (1) the diminution of adrenaline production in the adrenal medulla, or (2) the augmentation of adrenaline secretion of the adrenal. However, the possibility of the former is probably slight and the latter mechanism seems to be the case, since the section of the splanchnic nerves was proved in the present investigation to nullify the effect of insulin hypoglycemia upon the adrenaline content of the adrenal.

Thus it may be justified to deduce that the decrease in adrenaline content of the adrenal gland after insulin expresses the augmentation of adrenaline secretion, which is completely prevented by the splanchnicotomy. The adrenaline-secretory nerve fibers to the adrenal gland were proved to run mainly through the splanchnic nerves.

Summary

The noradrenaline and adrenaline content of the adrenal gland in toads was estimated by the permanganate method of Suzuki and Ozaki.

In normal toads the noradrenaline content was 0.110 (0.078–0.145) mg. per kg. of body weight and that of adrenaline was 0.187 (0.154–0.244) mg., the relative noradrenaline content being 37 (31–46) %.

Ten hours after insulin application the adrenaline content was found to be reduced markedly, while the noradrenaline content remained almost unaltered.

The decrease in the adrenaline content of the adrenal causable by insulin hypoglycemia was nullified by the section of the splanchnic nerves.

I am indebted to Prof. T. Suzuki for his interest during this investigation and for reading and criticizing this manuscript.

References

1) Suzuki, T. & Ozaki, T., Tohoku J. Exp. Med., 1951, **54**, 332; Ozaki, T., *ibid.*, 1954, **61**, 83.

2) Houssay, B. A., Gerschman, R. & Rapela, C. E., Compt. Rend. Soc. Biol., 1950, **144**, 1227.

3) Rapela, C. E. & Gordon, M., *ibid.*, 1956, **150**, 1290.
4) Hökfelt, B., Endocrinology, 1953, **53**, 536.
5) Burn, J. H., Hutcheon, D. E. & Parker, R. H. O., Brit. J. Pharmacol., 1950, **5**, 417.
6) Outschoorn, A. S., Nature, 1951, **167**, 722.

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Studies on Bile Pigment in Bile

Report I

Fluctuations of the Bilirubin Content in Liver Bile after the Operation for Various Biliary Tract Diseases

By

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After the operation of the biliary tract diseases, the bile output and the bilirubin content in the liver bile collected from the T-tube were measured continually for a certain duration. As a result, the author has found a certain tendency concerning the fluctuation of the bilirubin content in the liver bile which have been exhausted from the T-tube. The details are described in this paper.

Experimental

Materials and Methods

Twenty-five cases of various biliary tract diseases, into whose common duct the T-tube was inserted, were classified into three groups, viz.

Group I. 13 gallstone cases
Group II. 5 stoneless cholecystitis cases
Group III. 7 ascariasis of the biliary tract cases

Of these 25 cases, 22 were cholecystectomized and three were subjected to choledochostomy without cholecystectomy.

For experimental materials, the fresh liver bile from the T-tube was used, viz., the first day bile (24 hours after operation), the second day bile (the next 24 hours), the third day bile (the next 24 hours), and thus every 5th, 7th, 10th and 14th day bile was gathered from the T-tube in the sterile bottle. The total amount of these biles was measured respectively and a portion was used for determination of bilirubin.

The determination of the bilirubin content in bile was carried out after Malloy and Evelyn's method[1] (1937) ; viz. the bilirubin in bile was chemically changed into azobilirubin by means of diazotization and the bilirubin was colorimetrically determined by using Leitz's Rouy-photrometer with 535 mμ. The bilirubin content of the bile was much denser

than that of the serum, so the bile was diluted about 50–100 times with distilled water to suit the colorimetry. The details of the measurement are: each 4.0 m*l*. of distilled water and 5.0 m*l*. of absolute methyl alcohol were pipetted into two glass tubes, respectively. Then, 1.0 m*l*. of a newly prepared diazo reagent and 4.0 m*l*. of the sample of the diluted bile were added to each tube. By the colorimetry of both samples after the lapse of each 15 and 30 minutes after adding the diazo reagent, determination of direct reacting bilirubin (so-called conjugated form bilirubin)[2] and total bilirubin could be performed separately. The difference of the two should be the value of the indirect reacting bilirubin (so-called free form bilirubin).[2] In making the calibration curve for the photometric test, the pure bilirubin (Dai-ichi Kagaku) was applied.

Results

Group I. The determination of 13 cases in the gallstone group are tabulated in Table I. The bilirubin content in the first day bile was 67.0 mg/d*l* as its mean value; the second day bile 71.2 mg/d*l*, the third day bile 72.4 mg/d*l*, the 5th day bile 46.8 mg/d*l*, the 7th day bile 33.4 mg/d*l*, the 10th day bile 34.7 mg/d*l* and 14th day bile was 26.5 mg/d*l* respectively. The changes of these values are graphed in Fig. 1. This pattern shows the daily fluctuation of the bilirubin content in the liver bile after the operation, and it was recognized that the liver biles excreted from the T-tube had a stronger viscosity and a higher concentration for about five days after the operation.

In the course of 10 to 14 days after the operation, the bile became paler in color and also lower in viscosity. The determination result of the bilirubin content in liver bile taken in each 24 hours, are graphed in Fig. 4. In some cases the total bilirubin content of the first day recorded the highest value. In most cases, the total daily bilirubin content curve showed a high peak during the several days after the operation.

Group II. The determination results of five cases of the stoneless cholecystitis are given in Table II. In the first day bile, the bilirubin content was 147.6 mg/d*l* as its mean value; the second day bile 120.4 mg/d*l*, the third day bile 106.7 mg/d*l*, the fifth day bile 91.5 mg/d*l*, the 7th day bile 64.7 mg/d*l*, the 10th day bile 41.0 mg/d*l* and the 14th day bile was 36.6 mg/d*l* respectively. The changes of these values are graphed in Fig. 2. This pattern shows the same tendency as in Group I (Fig. 1). From the first to the fifth day after the operation, the bilirubin content was generally higher than in Group I. The total amount of the liver bile exhausted from the T-tube each 24 hours after the operation, is given in Table II. From the results obtained, the total bilirubin content in the liver bile each 24 hours is graphed in Fig. 5, which shows the same altera-

TABLE I

TABLE I

Bilirubin Content in Group I (Gallstone Cases)

No. of cases	Age	Sex	Diagnosis	1st day	2nd day	3rd day	5th day	7th day	10th day	14th day	Bilirubin content in bladder-bile
				Bilirubin content in liver-bile (mg/dl)							
				Amount of bile for each 24 hours (ml)							
3. K.O.	43	Female	Pigment chalk stones in the common duct	98.5 550	80.0 480	78.0 390	60.5 500	40.0 470	40.0 500	45.0 500	32.0
4. T.O.	18	"	ditto	72.0 400	95.5 390	106.0 290	90.0 520	80.5 240	48.0 360	— —	36.0
5. S.H.	41	"	ditto and atrophy of the gall-bladder	15.0 820	25.0 900	32.5 850	28.0 850	28.0 1100	26.0 750	16.5 1100	—
8. Y.B.	54	"	Pigment chalk stones in the common duct	160.0 280	110.0 800	170.5 700	90.0 700	15.0 700	40.0 800	— —	80.0
9. K.H.	31	Male	Pigment chalk stones in the liver	73.0 500	50.0 550	45.0 380	38.5 500	45.0 680	25.0 380	22.0 520	—
11. T.A.	41	Female	Pigment chalk stones in the bladder and duct	32.0 400	25.0 450	30.0 500	20.5 1250	15.0 750	10.5 700	— —	8.0
14. T.K.	51	"	Pigment chalk stones in the common duct	43.0 200	— —	67.5 450	15.0 500	63.0 350	62.0 150	— —	48.0
15. A.S.	38	"	Pigment chalk stone in the gall-bladder	35.0 300	50.0 300	40.5 310	40.0 120	35.0 120	25.0 550	15.5 270	—
16. S.H.	47	"	Pigment chalk stone in the papilla Vateri	55.0 450	60.0 300	50.5 280	40.5 120	20.2 700	30.0 550	18.0 650	—
19. M.H.	50	"	Cholesterol stone in the gall-bladder	182.0 120	200.0 100	153.0 80	Biliary hemorrhage	"	80.0 300	60.5 400	180.0
20. K.S.	29	"	Pigment chalk stones in the common duct	36.0 700	40.0 900	45.0 950	38.0 650	20.0 850	36.0 900	35.0 900	34.0
22. K.S.	19	Male	ditto	45.0 150	48.0 250	52.0 200	25.0 200	10.0 150	11.5 250	10.5 80	18.0
24. J.K.	30	"	ditto	25.0 200	— —	70.5 250	75.0 450	30.0 320	18.0 1000	16.0 1100	15.0
			Mean value	67.0	71.2	72.4	46.8	33.4	34.7	26.5	50.1

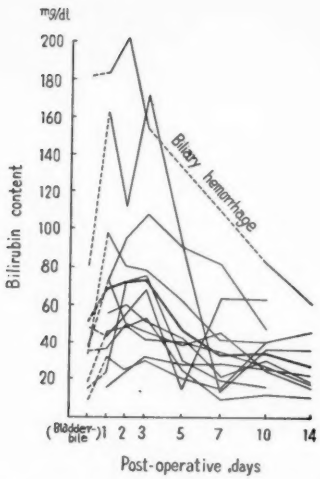

Fig. 1. Bilirubin content of Group I (gallstone cases).

tion as in Group I (Fig. 4). This pattern indicates a high peak for about five days after the operation. Ten to 14 days after the operation, the bile showed a comparatively lower concentration and viscosity.

Group III. The determination results of seven cases of ascariasis of the biliary tract are tabulated in Table III. In the first day bile, the bilirubin content was 99.3 mg/d*l* as its mean value; the second day bile 103.2 mg/d*l*, the third day bile 91.5 mg/d*l*, the 5th day bile 59.7 mg/d*l*, the 7th day bile 57.8 mg/d*l*, the 10th day bile 34.0 mg/d*l* and the 14th day bile was 35.0 mg/d*l* respectively. The fluctuation of these determination values is graphed in Fig. 3. This pattern indicates almost the same tendency as in Groups I and II (Figs. 1, 2).

The determination results of the amount of liver bile excreted in each 24 hours are graphed in Fig. 6. This pattern also shows the same trend as in Groups I and II (Figs. 4, 5).

Summary and Discussion

Considering the results mentioned above, it was noticed that the liver bile showed a yellowish deep brown color for several days after the operation, and with the lapse of 10 to 14 days after the operation, it became much dilute and light yellowish in color. This tendency is tentatively here named " the Post-operative Bilirubin Curve of Liver Bile ".

McMaster *et al.*[3] reported similar results using dogs with biliary fistula. They observed that the concentrated bile excreted from the biliary fistula for several days after being installed, because the surgical procedure

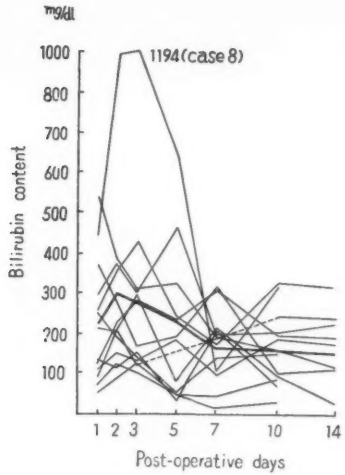

Fig. 4. Bilirubin content of total bile in each 24 hours (Group I)

TABLE II

Bilirubin Content in Group II (Stoneless Cholecystitis Cases)

No. of cases	Age	Sex	Diagnosis	1st day	2nd day	3rd day	5th day	7th day	10th day	14th day	Bilirubin content in bladder-bile
				Bilirubin content in liver-bile (mg/d/)							
				Amount of bile for each 24 hours (m/)							
7. N.N.	41	Male	Stoneless chronic cholecystitis	108.0 360	125.0 350	150.0 280	107.0 400	80.5 500	45.0 550	43.0 600	142.0
12. T.S.	66	"	ditto	45.0 400	52.0 210	60.5 170	45.0 150	43.0 200	28.0 110	— —	42.0
18. H.S.	36	Female	Papillitis	150.0 320	125.0 150	68.0 250	90.5 350	70.0 350	40.0 350	28.0 300	60.0
21. M.K.	26	"	ditto	85.0 70	120.0 300	135.0 300	55.0 250	50.0 200	38.0 50	30.0 200	—
23. R.I.	60	"	Stoneless chronic cholecystitis	350.0 200	180.0 150	120.0 250	160.0 500	80.0 350	54.0 200	45.5 300	40.0
			Mean value	147.6	120.4	106.7	91.5	64.7	41.0	36.6	71.0

would liberate the hemoglobin of extravasated blood in large quantity. On the other hand, Sakurai[4] stated that febrility caused the decrease of the bile excretion and the increase of the bilirubin content in bile. Takano[5] recognized that, in the early stage of the temperature rising, the bile excretion and the organic substance in bile increase temporarily. Matsuo[6]

Fig. 2

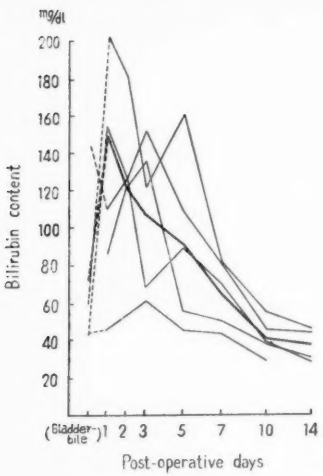

Fig. 5

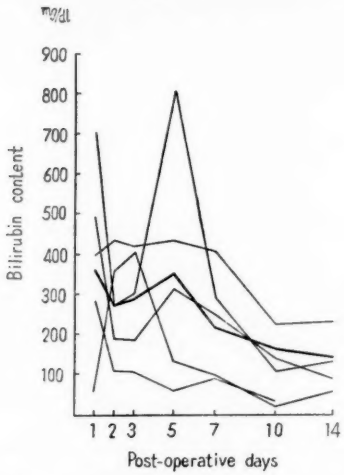

Fig. 2. Bilirubin content curve of Group II (stoneless cholecystitis group).
Fig. 5. Bilirubin content of total bile in each 24 hours (Group II).

described that the excreting function of various pigments into the bile would alter with the season, and Nakagawa[7] noticed the relation between the liver function and the pigment formation.

Then, the author examined in vain the correlation between " the Bilirubin Curve " and various clinical manifestations in the individual, especially the interrelation between this curve and cholecystectomy or the functional conditions of the liver. Further investigation may be required to solve this problem. In any way, the tendency to excrete more concentrated bile after the operation as shown in " the Bilirubin Curve " may have a significance concerning the propriety of detaining the T-tube in the common duct at the time of the operation.

Conclusion

The author determined the biliribin content in the liver bile, which was exhausted from the T-tube detained in the common duct after the operation for various biliary tract diseases. The 25 cases comprised, 13 of gallstone, five cases of stoneless cholecystitis and seven of ascariasis of the biliary tract. As a result, it was recognized that the thick liver bile excreted in each case from the T-tube during several days after the operation. After 10 to 14 days following the operation, the liver bile showed a comparatively lower concentration in most of the cases. The author named this tendency in the fluctuation of the bilirubin content, " the Post-operative

TABLE III

Bilirubin Content in Group III (Cases of Ascariasis of the Biliary Tract)

No. of cases	Age	Sex	Diagnosis	1st day	2nd day	3rd day	5th day	7th day	10th day	14th day	Bilrubin content in bladder-bile
				Bilirubin content in liver-bile (mg/dl)							
				Amount of biles for each 24 hours (ml)							
1. C.H.	40	Female	Ascariasis of the biliary tract	250.0 180	194.0 210	196.0 220	115.5 400	110.0 380	64.0 350	— —	105.0
2. S.H.	7	Male	ditto	80.0 90	100.0 190	75.5 200	60.0 210	30.0 200	25.0 230	— —	74.0
6. H.O.	11	"	ditto	124.0 205	110.0 250	65.0 160	40.0 250	35.5 300	— —	— —	125.0
10. M.K.	26	Female	ditto and chronic cholecystitis	32.0 500	90.5 300	92.0 660	73.0 650	60.0 300	30.5 460	— —	178.0
13. T.T.	43	"	Ascariasis of the biliary tract	108.0 200	98.0 500	90.5 320	55.0 350	50.0 460	— —	55.0 200	60.5
17. K.K.	8	"	ditto	60.5 400	50.0 200	46.5 180	35.0 250	34.0 200	20.5 350	15.0 400	50.0
25. H.M.	64	"	ditto and cholecysto-cholangitis acuta	40.5 400	80.0 420	75.0 350	40.0 310	85.0 320	30.0 500	— —	320.0
			Mean value	99.3	103.2	91.5	59.7	57.8	34.0	35.0	130.4

Fig. 3

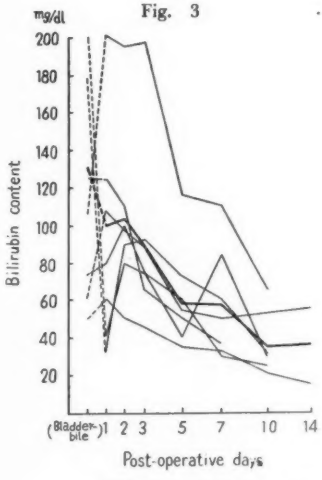

Fig. 6

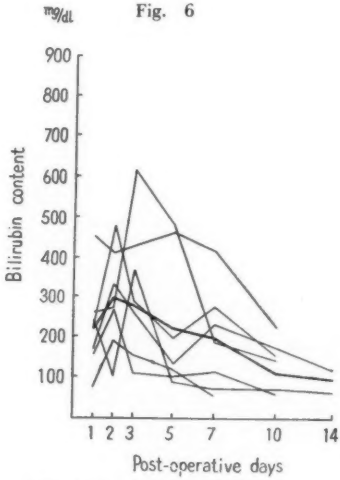

Fig. 3. Bilirubin content curve of Group III (group of ascariasis of the biliary tract).
Fig. 6. Bilirubin content of total bile in each 24 hours (Group III).

Bilirubin Curve of Liver Bile ". But the reason for this tendency has not yet been clarified.

The author appreciates the kind help and assistance of Professor Noboru Hiyama and Dr. Masahiro Maki of the Department of Biochemistry with regard to the present experiment.

References

1) Malloy & Evelyn, J. Biol. Chem., 1937, **119**, 481.
2) Satô, Hirosaki Med. J., 1955, **6**, 87.
3) McMaster & Rous, J. Exper. Med., 1923, **37**, 395.
4) Sakurai, Tokyo-Igakkai-Zasshi (Jap.), 1926, **40**, 117.
5) Takano, Jikken-Shokakibyo-Gaku (Jap.), 1927, **1**, 1199.
6) Matsuo, Biologische Untersuchungen über Farbstoffe, Vol. 2, Kyoto, 1935, 803.
7) Nakagawa, J. J. S. I. M., 1933, **21**, 71.

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Studies on Bile Pigment in Bile

Report II

Clinical Consideration on the Bilirubin Content of Bile in Patients with Various Biliary Tract Diseases

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(Received for publication, June 7, 1958)

Concerning the physical and chemical properties of the bile in the pathologic states, Maruyama,[1] Hashimoto,[2] Narumi,[3] and Tsushima[4] of our clinic have carried out many investigations.

The author, as a series of these studies, has determined the bilirubin content in the bile in the clinical cases of various biliary tract diseases with the results given herein.

Experimental

Materials and Methods

The experiment was made on 52 laparotomized cases classified into four groups: 1) 11 gallstone cases (8 of pigment stone and 3 of cholesterol stone), 2) five cases of so-called stoneless cholecystitis, 3) seven cases of ascariasis of the biliary tract, and 4) 26 cases of non-biliary tract diseases as control. The gall-bladder and common duct biles of these cases were collected by puncture of them at the time of the operation. Of them, eight cases (5 of gallstone, 2 of stoneless cholecystitis, and 1 of ascariasis of the biliary tract) were used for the experiment of the duct bile.

The determination of the bilirubin content in the bile was carried out according to Malloy and Evelyn's method[5] by using Leitz's Rouy-photrometer, described in the previous paper[6]. When the bile was too thick, it was diluted with the distilled water. In the present paper, not only the total bilirubin content, but also the direct reacting bilirubin (so-called conjugated form bilirubin[7]) and the indirect reacting bilirubin (so-called free form bilirubin[7]) were estimated.

Results

I. Bilirubin content in gall-bladder bile

i) Control group

The bilirubin content in the bile of 26 cases as control group is shown in Table I. The total bilirubin content was 85.5–1890.0 mg/d*l*,

TABLE I

Bilirubin Content in the Gall-bladder Bile of 26 Cases of Non-biliary Tract Diseases

No. of cases	Patient Age	Sex	Diagnosis	Biririubin content (mg/d*l*)			Ratio of conjugated form to total bilirubin (%)
				Conjugated form	Free form	Total bilirubin	
3. S. O.	40	Male	Gastritis chronica	1385.0	5.0	1390.0	99.6
4. A. H.	33	Female	Enteroptosis	650.0	70.0	720.0	90.2
5. C. M.	27	"	Intestinal stenosis	1145.0	260.0	1405.0	81.5
6. T. K.	47	"	Stomach cancer	820.0	92.0	912.0	89.9
7. H. T.	30	Male	Enteroptosis	1375.0	110.0	1485.0	89.8
8. A. H.	23	Female	ditto	205.0	15.0	220.0	93.2
9. T. S.	24	"	ditto	1067.5	160.0	1227.5	87.1
10. T. K.	35	Male	ditto	1605.0	285.0	1890.0	84.9
13. K. M.	2	"	Foreign body in the stomach	138.5	3.5	142.0	97.5
14. T. I.	54	Female	Stomach cancer	77.0	12.0	89.0	86.6
15. J. K.	36	Male	Enteroptosis	685.0	65.0	750.0	91.3
16. T. N.	45	"	ditto	660.0	60.0	720.0	91.8
17. K. H.	50	"	ditto	1240.0	230.0	1470.0	84.4
18. S. K.	28	Female	ditto	540.0	60.0	600.0	90.0
20. K. K.	46	"	ditto	380.0	30.0	410.0	92.7
21. S. H.	36	"	ditto	1070.0	170.0	1240.0	86.3
22. S. K.	55	Male	ditto	865.0	135.0	1000.0	86.5
24. C. S.	23	Female	ditto	—	—	142.0	—
25. Y. M.	49	Male	Duodenal ulcer	81.5	4.0	85.5	95.3

TABLE I—*Concluded*

No. of cases	Patient Age	Sex	Diagnosis	Birirubin content (mg/d*l*)			Ratio of conjugated form to total bilirubin (%)
				Conjugated form	Free form	Total bilirubin	
26. K. K.	43	Female	Stomach cancer	710.0	115.0	825.0	86.1
27. Y. K.	46	"	ditto	260.0	10.0	270.0	96.3
29. K. Y.	33	"	Enteroptosis	1420.0	205.0	1625.0	87.4
30. K. A.	18	"	Ileocaecal tuberculosis	280.0	10.0	290.0	96.6
31. Y. N.	43	Male	Stomach cancer	600.0	110.0	710.0	84.5
32. S. S.	75	"	Peritonitis carcinomatosa	685.0	20.0	705.0	97.2
38. K. H.	54	Female	Cancer of the pancreas	415.0	35.0	450.0	92.2
			Mean value	734.4	90.9	799.0	90.4

and its mean value 799.0 mg/d*l*, the content of the conjugated form 77.0–1605.0 mg/d*l*, and its mean 734.4 mg/d*l*, while the free form 3.5–285.0 mg/d*l*, and its mean 90.9 mg/d*l*.

Toida[8] determined following Hoppe-Seyler's method, the mucin and bile pigment content in the gall-bladder bile in 16 healthy persons, of which the mean was reported as 944 mg/d*l*. This value is greater than the author's. On the other hand, as for the Caucasian, owing to Frerichs's and Hammarsten's reports[9], the bilirubin content in the gall-bladder bile are respectively 2820 mg/d*l* and 4320 mg/d*l*, either of which shows a higher value than that of any Japanese.

The ratio of the content of the conjugated form to the total bilirubin content was 81.5–99.6 per cent, and its mean was 90.4 per cent. Consequently, it may be found that the conjugated form holds the greater part of the bilirubin content in bile. This phenomena shows the trend similar to that of the experiment performed by the use of paperchromatography[7]. The appearance of the gall-bladder bile in the control group was generally yellowish dark brown in color, and showed strong viscosity.

ii) *Gallstone group*

Among the 11 cases of the gallstone group, eight of pigment stone and three of cholesterol stone were classified for the purpose of the observation. The results of the determination for the bilirubin content in the gall-bladder bile are given in Table II. First, the total bilirubin content in the gall-bladder bile in the case of pigment stone was

TABLE II

Bilirubin Content in the Gall-bladder Bile of 11 Cases of Gallstone, of Which Eight Cases of Pigment Stone and Three Cases of Cholesterol Stone

No. of cases	Patient Age	Sex	Diagnosis	Bilirubin content (mg/dl) Conjugated form	Free form	Total bilirubin	Ratio of conjugated form to total bilirubin (%)	Bacteria in the bile
Pigment stone cases								
11. K.O.	43	Female	Pigment chalk stones in the common duct	26.0	6.0	32.0	81.3	Bac. coli (+)
12. T.O.	18	"	ditto	33.0	3.0	36.0	91.6	Bac. coli (+)
33. Y.B.	54	"	Pigment chalk stones in the gall-bladder and common duct	77.0	3.0	80.0	96.3	Bac. coli (+)
35. T.A.	41	"	ditto	7.0	1.0	8.0	87.5	(−)
39. J.K.	30	Male	Pigment chalk stones in the common duct	11.5	3.5	15.0	76.7	Bac. coli (+)
41. K.H.	19	"	ditto	15.5	3.5	19.0	81.6	Bac. paracoli (+)
42. K.S.	29	Female	ditto	30.0	2.0	32.0	93.8	Bac. coli (+)
50. T.K.	51	"	ditto	46.0	2.0	48.0	95.8	Bac. coli (+)
			Mean value	30.8	3.0	33.8	88.1	Bac. coli (+)
Cholesterol stone cases								
40. M.H.	50	Female	Cholesterol stones in the gall-bladder	173.5	6.5	180.0	96.4	Bac. proteus (+)
43. S.K.	36	"	ditto	82.0	2.0	84.0	97.6	(−)
51. K.M.	39	Male	ditto	125.0	4.0	129.0	96.1	(−)
			Mean value	126.8	4.2	131.0	96.7	

8.0–80.0 mg/dl, and its mean 33.8 mg/dl, while the content of the conjugated form 7.0–77.0 mg/dl, and its mean 30.8 mg/dl. The ratio of the content of the conjugated form to the total bilirubin content was 81.3–96.3 per cent and its mean 88.1 per cent. Next, the total bilirubin content in the gall-bladder bile in the case of cholesterol stone was 84.0–180.0 mg/dl, and its mean 131.0 mg/dl, while the content of the conjugated form 82.0–173.5 mg/dl, and its mean 126.8 mg/dl, and the content of the free form

2.0–6.5 mg/d*l*, and its mean 4.2 mg/d*l*. The ratio of the content of the conjugated form to the total bilirubin content was 96.1–97.6 per cent, and its mean 96.7 per cent.

Considering the results mentioned above, with the cases of the gall-stone group, their bilirubin content in the gall-bladder bile, in comparison with the control group seemed to show an excessively small value. This trend appeared stronger in the case of pigment stone than in that of cholesterol stone. The gall-bladder bile found in the cases of pigment stone was mostly light yellowish green in color and its viscosity seemed to be remarkably low in general. As for the bacterial culture, colibacilli were found in seven out of eight cases. On the other hand, the properties of the gall-bladder bile taken from the cases of cholesterol stone was a little thick in appearance in comparison with that of pigment stone cases. As for the bacilli in bile, proteus bacteria could be found only in one case.

iii) *Stoneless cholecystitis group*

The results of measurement in five cases of stoneless cholecystitis are shown in Table III. The total bilirubin content was 40.0–142.0 mg/d*l*,

TABLE III

Bilirubin Content in the Gall-bladder Bile of Five Cases of Stoneless Cholecystitis

No. of cases	Patient Age	Sex	Diagnosis	Bilirubin content (mg/d*l*)				Bacteria in the bile
				Conjugated form	Free form	Total bilirubin	Ratio of conjugated form to total bilirubin (%)	
23. K.Y.	34	Male	Stoneless chronic cholecystitis	123.0	17.0	140.0	88.0	
28. N.N.	41	"	ditto	130.0	12.0	142.0	91.5	Bac. coli (+)
36. T.S.	66	"	ditto	38.0	4.0	42.0	90.4	Bac. coli (+)
46. R.I.	60	Female	Papillitis	37.5	2.5	40.0	93.8	(−)
47. H.S.	36	"	ditto	55.5	4.5	60.0	92.5	
			Mean Value	76.8	8.0	84.8	91.2	

and its mean 84.8 mg/d*l*, while the content of the conjugated form 37.5–130.0 mg/d*l*, and its mean 76.8 mg/d*l*. The content of the free form was 2.5–17.0 mg/d*l*, and its mean 8.0 mg/d*l*. The ratio of the content of the conjugated form to the total bilirubin content was 88.0–93.8 per cent, and its mean 91.2 per cent.

From the results of these determinations, it was estimated that the bilirubin content in the gall-bladder bile of the cases of stoneless cholecystitis was greater than that of pigment stone cases. However, as compared with the bilirubin content in the cases of cholesterol stone, the above results show a little lower value, and it is also seemingly extremely low comparing with that of the control cases. The density of gall-bladder bile of the cases of stoneless cholecystitis, comparing with that of pigment stone, was found to be fairly high. But, in comparison with the control group, the density and viscosity seemed to be remarkably low. With the results of the bacterial culture of the biles in the cases of stoneless cholecystitis, colibacilli were found in two out of five cases.

iv) Group of ascariasis of the biliary tract

The results of seven cases of ascariasis of the biliary tract are shown in Table IV. The total bilirubin content was 50.0–320.0 mg/d*l*, and its

TABLE IV

Bilirubin Content in the Gall-bladder Bile of Seven Cases of Ascariasis of the Biliary Tract

No. of cases	Patient Age	Sex	Diagnosis	Bilirubin content (mg/d*l*) Conjugated form	Free form	Total bilirubin	Ratio of conjugated form to total bilirubin (%)	Bacteria in the bile
1. C.H.	40	Female	Ascariasis of the biliary tract	103.0	2.0	105.0	98.1	(−)
2. S.H.	7	Male	ditto	72.5	1.5	74.0	98.0	Bac. coli (+)
19. H.O.	11	"	ditto	117.5	7.5	125.0	94.0	(−)
34. M.K.	26	Female	ditto	162.0	16.0	178.0	91.0	(−)
37. M.K.	43	"	ditto and cholangitis acuta	53.0	7.5	60.5	87.6	Bac. coli (+)
44. F.M.	64	"	ditto and cholecystitis acuta	234.0	86.0	320.0	73.1	Bac. coli (+)
45. K.K.	8	"	Ascariasis of the biliary tract	—	—	50.0	—	
			Mean value	123.7	20.1	130.4	90.3	

mean 130.4 mg/d*l*, while the content of the conjugated form 53.0–234.0 mg/d*l*, and its mean 123.7 mg/d*l*. The content of the free form was 1.5–86.0 mg/d*l*, and its mean 20.1 mg/d*l*. The ratio of the content of the conjugated form to the total bilirubin content was 73.1–98.1 per cent, and its mean 90.3 per cent.

Considering the above results, the bilirubin content in the gall-

bladder bile in the ascariasis of the biliary tract shows a much higher value than that of the pigment stone cases. But it was almost the same as that of the bilirubin content in the cholesterol stone cases. It had a little higher value than that of the bilirubin content in the bile of the stoneless cholecystitis group, although, comparing with the value of control group, it was considerably lower. The gall-bladder bile found in the ascariasis of the biliary tract was generally deeper in color than that of pigment stone cases or stoneless cholecystitis cases. In fresh cases (No. 26), the viscosity of the bladder bile seemed to be fairly strong. The bacterial culture of the bile revealed the existence of colibacilli in three out of seven cases.

II. Bilirubin content in the duct bile.

As stated previously, the duct bile was obtained by direct puncture of eight cases in all, namely, five of gallstone, two of stonless cholecystitis and one of ascariasis of the biliary tract. The results of determination of

TABLE V

Bilirubin Content in the Common Duct Bile of Eight Cases of Biliary Tract Disease

No. of cases	Patient Age	Sex	Diagnonis	Bilirubin content (mg/dl) Conjugated form	Free form	Total bilirubin	Ratio of conjugated form to total bilirubin (%)	Bacteria in the bile
12. T.O.	18	Female	Pigment chalk stones in the common duct	33.1	7.0	40.0	82.5	Bac. coli (++)
48. S.H.	41	"	ditto and atrophy of the gall-bladder	12.0	2.5	14.5	82.8	Bac. coli (+)
28. N.N.	41	Male	Stoneless chronic cholecystitis	171.0	28.0	119.0	85.9	Bac. coli (+)
33. Y.B.	54	Female	Pigment chalk stones in the common duct and gall-bladder	76.0	5.0	81.0	93.8	Bac. coli (+)
49. K.H.	31	Male	Liver stones and atrophy of the gall-bladder	36.0	2.5	38.5	93.5	Bac. coli (+)
37. T.T.	43	Female	Ascariasis of the biliary tract	54.0	8.0	62.0	87.1	Bac. coli (+)
50. T.K.	51	"	Pigment chalk stones in the common duct	46.0	2.0	48.0	95.8	Bac. coli (+)
52. A.I.	46	Male	Secondary biliary dyskinesia after cholecystectomy	88.5	6.0	94.5	93.7	(−)
			Mean value	64.6	7.6	72.2	89.4	

the bilirubin content in the duct bile are tabulated in Table V. Their

total content was 14.5–119.0 mg/d*l*, and its mean 72.2 mg/d*l*, while the bilirubin content of the conjugated form 12.0–171.0 mg/d*l*, its mean 64.6 mg/d*l* and the bilirubin content of the free form 2.0–28.0 mg/d*l*, and its mean 7.6 mg/d*l*. The ratio of the content of the conjugated form to the total bilirubin content was 82.5–93.8 per cent, and its mean 89.4 per cent.

According to Miyake and Ishiyama[10], the mucin and bile pigment content in liver bile of the Japanese was 91.4–412.6 mg/d*l*, and its mean 216.4 mg/d*l*, while that of the Caucasian described by Hammarsten[9] was 13.0–910.0 mg/d*l*, and its mean 317.8 mg/d*l*. The results of determination of the duct bile in the pathologic state by the author showed a remarkably small value in comparison with the mentioned reports. From these facts, the bilirubin content in the duct bile, as in the gall-bladder bile, was recognized to have general characteristic of low concentration in the biliary tract diseases.

The appearance of the duct bile in pigment stone cases was found to be much lighter than that of the gall-bladder bile. With the case No. 41, the duct bile was almost water-like and it was found to be nearly greenish in color tone. As for result of the bacterial culture of these biles, colibacilli and other bacteria were found out in seven out of eight cases.

Summary and Discussion

As described above, it is estimated that the bilirubin content in the gall-bladder bile of the Japanese shows a smaller value in comparison with that of the Caucasian. With pigment stone cases, the mean value of determination was 33.8 mg/d*l*, while in cholesterol stone cases, its mean was 131.0 mg/d*l* respectively, showing lower concentrations than those of the control group. As for the results of determination of stoneless cholecystitis cases, its mean was 84.8 mg/d*l*. In contrast to that of pigment stone cases it showed a rather high value, and a lower value than that of cholesterol stone cases. Comparing with that of the control group, it showed a lower concentration. As for the results of determination in the ascariasis of the biliary tract, its mean was 130.4 mg/d*l*, as against those of pigment stone cases and stoneless cholecystitis cases, showing a fairly high concentration. The bilirubin content in the common duct bile also decreased, its mean value being 72.2 mg/d*l*.

In view of these facts, with the cases of the biliary tract diseases, it may be said that the bilirubin content in the gall-bladder biles and common duct biles inclined to decrease respectively. It is recognized that the conjugated form bilirubin occupies nearly 90 per cent of the total bilirubin in bile even in the diseased cases.

Tsushima[4] of our clinic, in consequence of determination of the cholesterol content in the bile found in cases of various biliary tract diseases,

mentioned that the cholesterol content had some correlation with the liver function. But such a relationship between the functional disorders of the liver and the bilirubin content in the bile could not be derived from my results.

Conclusion

The bilirubin content in the clinical cases (11 of gallstone, 5 of stoneless cholecystitis and 7 of ascariasis of the biliary tract) were determined. The results obtained indicate that:—The bilirubin content in the bile in pathologic states, comparing with that of the non-biliary tract diseases, was recognized to remarkably decrease and this trend was peculiarly obvious in pigment stone cases.

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References

1) Maruyama, To be published in this Journal.
2) Hashimoto, Hirosaki Med. J., 1956, **7**, 323.
3) Narumi, *ibid.*, 1957, **8**, 678.
4) Tsushima, *ibid.*, 1958, **9**, 281.
5) Malloy & Evelyn, J. Biol. Chem., 1937, **119**, 481.
6) Satô, Published in this Journal as Report I.
7) Satô, Hirosaki Med. J., 1955, **6**, 87.
8) Toida, J.J.S.S., 1914, **17**, 147.
9) Frerichs & Hammarsten, Cited from Sobotka, Physiological chemistry of the bile. Baltimore, W. Wilkins, 1937, 19.
10) Miyake & Ishiyama, Naigai-kyoiki-mondai to-shi-te-no tansekisho (Jap.), Tokyo, Kokuseido, 1927, 66.

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Studies on Bile Pigment in Bile

Report III

Bilirubin Content of Bile in Dogs with Pathologically Changed Biliary Tract

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After the previous experiment[1] which dealt with the human bile, the bilirubin content in the dog bile was determined, when the pathologic states had been induced experimentally in the biliary tract of the dog.

Experimental

Materials and Methods

The dogs used in the experiment were 13 healthy ones, weighing 7 to 14 kg. These had been bred on the hospital leftover until the experiment was performed, and they were divided into the three experimental groups:

Group I. With a transduodeno-papillary insertion of Nelaton's catheter (No. 3) into the common duct for 2 or 3 hours.

Group II. With the introduction of ascaris egg suspension from Nelaton's catheter, after the same procedure as employed with Group I.

Group III. With a loose ligature of the cystic duct after the same procedure as with Group II.

Prior to the experiment, every dog was kept from food at least four to six hours, and 1.0 m*l* per kg. of 4 per cent morphine hydrochloride was used for the basal narcosis in combination with 0.5 per cent procaine hydrochloride for local anesthesia, then the laparotomy was applied to each one. By the direct puncture of the gall-bladder, 1.0 m*l* to 2.0 m*l* of the bile was obtained aseptically. With Group I, Nelaton's catheter (No. 3) was inserted into the common duct to the depth of about 2.0 cm. from the papilla of Vater, which was taken out after two to three hours keeping. With Group II, after the procedure adopted with Group I, 2.0 m*l* of ascaris egg suspension was introduced through the catheter according to Atsumi's method.[2] With Group III, after the same procedure as taken with

Group II, a loose ligature of the cystic duct was applied so that stasis might be occasioned in the bladder without a complete closure of the duct. After the lapse of the stated time, the animals were re-operated upon and in most cases their gall-bladders were removed. From them bile was obtained aseptically for experimental purposes. As a method used for the determination of the bilirubin content in dog bile, the same one as described in the previous paper (Report I)[3] was employed. But with the dog bile obtained, the direct reacting bilirubin (so-called conjugated form bilirubin)[4] could not be measured owing to the reaction mixture in the test tube becoming turbid in most cases at the time of the colorimetric determination. This turbidity of the reaction mixture was estimated to be due to the influence of lipid or similar substances in the bile. In the determination of the total bilirubin content, however, the obstacle mentioned above was almost ruled out. Accordingly, it was possible to observe the bilirubin content in the gall-bladder bile of the dogs only as total bilirubin.

Results

Group I. A group with a transduodeno-papillary insertion of Nelaton's catheter (No. 3) into the common duct for two or three hours.

Fukushima[5] and Mori[6] of our clinic, according to the procedure mentioned above, recognized that some pathological changes of the papilla of Vater, such as edema and proliferation of the connective tissue were the cause of the rising of intraductal pressure and dilatation of the common duct. On that occasion, the bilirubin content in the gall-bladder bile was determined. The results obtained from the five dogs are tabulated in Table I. The bilirubin content in the bladder bile of the dog Nos. 3 and

TABLE I

A Group with a Transduodeno-papillary Insertion of Nelaton's Catheter (No. 3) into the Common Duct for Two or Three Hours

No. of dog	Sex	Body weight (kg)	Pre-operative total bilirubin content (mg/dl)	Post-operative days	Post-operative total bilirubin content (mg/dl)	Quantity of increase or decrease (mg/dl)	Ratio of increase or decrease (%)
3	♀	12	38.0	6	70.5	+32.5	+ 86
4	♀	8	36.0	7	43.0	+ 7.0	+ 19
6	♀	7	146.0	10	213.0	+67.0	+ 46
7	♂	10	34.5	14	97.5	+63.0	+183
11	♀	10	60.5	34	57.0	− 3.5	− 6

4, which were examined at the end of a week after the first operation, were

70.5 mg/d*l* and 43.0 mg/d*l* respectively. The ratio of increase accounting from the pre-operative determination value was 86 per cent and 19 per cent, respectively. The results of the determination of the dogs Nos. 6 and 7, which were examined at the end of 10 to 14 days after the first operation, were 213.0 mg/d*l*, and 97.5 mg/d*l* individually viewed. The ratio of increase, in view of the pre-operation value was 46 per cent and 183 per cent respectively, while the dog No. 11 recorded, 34 days after the first operation, 57.0 mg/d*l* and in this case, the ratio of decrease was 6 per cent in view of the value in the pre-operation.

It is estimated that, with Group I the bilirubin content in gall-bladder, as a whole, showed a tendency to increase until about two weeks after the operation. The color tone of the bile in the experimental cases was respectively almost similar as that of the pre-operation within seven days after the first operation (Nos. 3 and 4), and neither turbidity nor biliary sand was observed in either biles. With Nos. 6 and 7 after the elapse of 10 and 14 days, their biles were more yellowish brown than that of the pre-operation, and their viscosity seemed to have increased. As for the experimental dog No. 11, after the elapse of over thirty days, it was found to have turned a little greenish brown in color.

Group II. A group with the introduction of ascaris egg suspension from Nelaton's catheter, after the same procedure as employed with Group I.

The results of the determination of the bilirubin content in the gall-bladder bile of four dogs are presented in Table II. The bilirubin content in the

TABLE II

A Group with the Introduction of Ascaris Egg Suspension through Nelaton's Catheter, after the Same Procedure as Employed with Group I

No. of dog	Sex	Body weight (kg)	Pre-operative total bilirubin content (mg/d*l*)	Post-operative days	Post-operative total bilirubin content (mg/d*l*)	Quantity of increase or decrease (mg/d*l*)	Ratio of increase or decrease (%)
12	♂	12	124.0	2	84.5	−39.5	−32
16	♀	14	50.5	7	31.0	−19.5	−39
21	♂	8	132.0	29	129.0	− 3.0	− 2
22	♀	10	84.0	29	76.5	− 7.5	− 9

gall-bladder bile of the two dogs (Nos. 12 and 16), was respectively 84.5 mg/d*l* and 31.0 mg/d*l* within one week after the first operation ; the ratio of the decrease was 32 per cent (No. 12) and 39 per cent (No. 16) in view of their values of the pre-operation. The results of determination with Nos. 21 and 22 after 29 days of the first operation, were 129.0 mg/d*l* and

76.5 mg/d*l*, indicating that the ratio of the decrease was 2 per cent and 9 per cent respectively. The bile in Nos. 12 and 16 was greenish brown in color, and their viscosity seemed to be lower. With Nos. 21 and 22, the color of their bile was similar to that of the pre-operation, but they were a little more greenish than the latter.

Group III. A group with a loose ligature of the cystic duct after the same procedure as taken with Group II.

The results of the determination of the bilirubin content in the gall-bladder bile found in four dogs that received the above operation are shown in Table III. The bilirubin content in the gall-bladder bile of the

TABLE III

A Group with a Loose Ligature of the Cystic Duct after the Same Procedure as Taken with Group II

No. of dog	Sex	Body weight (kg)	Pre-operative total bilirubin content (mg/d*l*)	Post-operative days	Post-operative total bilirubin content (mg/d*l*)	Quantity of increase or decrease (mg/d*l*)	Ratio of increase or decrease (%)
26	♀	10	122.0	9	27.0	− 95.0	−78
28	♀	13	152.0	9	36.0	−116.0	−76
29	♂	8	86.0	10	20.5	− 66.0	−76
30	♂	12	102.0	11	17.0	− 85.0	−85

dogs Nos. 26 and 28, about 10 days after the operation, was 27.0 mg/d*l* and 36.0 mg/d*l*; the ratio of decrease was 78 per cent and 76 per cent respectively. The determination value of the dogs Nos. 29 and 30 was 20.5 mg/d*l* and 17.0 mg/d*l*; the ratio of decrease was 76 per cent and 85 per cent respectively. Each showed a remarkable decrease. As for the color tone of the bile of the dogs of Group III, it was greenish yellow, with Nos. 26 and 28 and greenish light brown with Nos. 29 and 30. These phenomena seem to rise from the fact that the bilirubin in the gall-bladder bile changed into biliverdin and other derivatives, perhaps due to the infection, the stasis and the admixture of organic matter. The viscosity of these biles was found to be in all cases remarkably low in value.

SUMMARY AND DISCUSSION

The results mentioned above showed that the bilirubin content in the bladder bile of dogs somewhat increase within two weeks after the papilla of Vater had been stimulated and secondary dyskinesia had been induced. And it seemed to increase gradually in its content after one month. As regards the cases with which infection and stasis had been caused in the biliary tract by the introduction of ascaris egg suspension

and a loose ligature of the cystic duct, the decrease of the bilirubin content was more rapid, viz. within one week. The color of the bile also changed into a greenish brown on this occasion, and it seemed much lower in its viscosity.

Miyake[7] reported that the bilirubin content in the bile showed a tendency to increase by the infusion of the colibacilli suspension into the biliary tract from the biliary fistula installed in the dog. And the pigment stones are apt to form because of the increase of the bilirubin content in the infected bile. On the contrary, in my experiment as described in the previous paper,[1] the bilirubin content in the bile showed a marked decrease with the clinical cases of the biliary tract diseases, almost all of these cases proving to have colibacilli in the bile.

In the present experiment, with dogs, the bilirubin content also showed a tendency to decrease remarkably after the manipulation both with Groups II and III. This fact may confirm the results obtained with the clinical cases described in Report II.[1] As for the appearance of the bile, a yellowish brown, the pre-operative color tone changed into a greenish brown or yellowish green. This phenomenon is considered to be based on the change of bilirubin into biliverdin and other derivatives, caused by stasis and infection of the bile. From the fact mentioned above, it may not be said that only the quantitative increase of bilirubin in the bile is an important factor, as Miyake[7] described, in the formation of bilirubin chalk stone.

Conclusion

In Report II,[1] the author described that the bilirubin content in various kinds of pathologic human biles was much less than that of healthy human biles. In the present study, it has been confirmed that a similar tendency is shown in the pathologic dogs biles. It has been proved through the animal experiment that the stasis and infection of the biliary tract will accelerate the lessening of the bilirubin content in the bile.

References

1) Satô, Published in this Journal as Report II.
2) Atsumi, Hirosaki Med. J., 1956, **7**, 1.
3) Satô, Published in this Journal as Report I.
4) Satô, Hirosaki Med. J., 1955, **6**, 87.
5) Fukushima, *ibid.*, 1955, **6**, 351.
6) Mori, Histo-pathological studies on the papillitis of dogs induced by mechanical stimulation, to be published in Hirosaki Med J., 1958.
7) Miyake, Hukuoka-Ikadaigaku-Zasshi (Jap.) 1933, **1**, 88.

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Studies on Bile Pigment in Bile

Report IV

Experimental Study on Bilirubin Content of Bile in Dogs fed on High or Low Protein

By

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(Received for publication, June 7, 1958)

In the Report II,[1] the author described that the bilirubin content in bile of the Japanese was remarkably small in quantity in comparison with the Caucasian. This may not be based on the difference of the race, but on the difference of the food. The scope of the present experiment is to know the effect of the high and low protein food upon the bilirubin excretion in dog bile.

Experimental

Materials and Methods

For the experiment, 17 healthy dogs weighing 6–14 kg. were used. These dogs had been fed on leavings in the hospital for about a week. They were divided in two groups without discrimination and to 8 dogs (Group I) was given the high protein food, and to 9 dogs (Group II) was given the low protein, respectively.

The ingredients of the high protein food given to per 10 kg. body weight a day were (Table I), 49.6 g. of protein, 5.0 g. of fat, 189.5 g. of carbohydrate, the total calorie being 1006.2, while those of the low protein food given were (Table II), 20.4 g. of protein, 5.1 g. of fat, 220.3 g. of carbohydrate, the total calorie being 1009.6.

The foods of both group were almost the same with regard to the quantities of fat, carbohydrate and the total calorie given, but the quantity of protein was different; the high protein food was prepared so that it might contain twice as much as the low protein food.

These two kinds of food were prepared with great care and precision at the culinary department of this hospital. They were given to the dogs once a day. No other nourishment was given to them except for a sufficient quantity of drinking water containing a little salt. The duration

TABLE I

Composition of High Protein Food (Per 10 kg. Body Weight a Day)

	Weight (g.)	Protein (g.)	Fat (g.)	Carbohydrate (g.)	Calorie
Boiled rice	500.0	13.4	1.7	162.0	720.0
Bean-mash	40.0	4.8	1.8	6.6	63.2
Cooked fish	100.0	17.5	1.1	0	80.0
Salt	A little				
Defatted milk-powder	40.0	13.9	0.4	20.9	143.0
Total	680.0	49.6	5.0	189.5	1006.2

TABLE II

Composition of Low Protein Food (Per 10 kg. Body Weight a Day)

	Weight (g.)	Protein (g.)	Fat (g.)	Carbohydrate (g.)	Calorie
Boiled rice	650.0	18.0	2.2	217.0	960.0
Bean-mash	20.0	2.4	0.9	3.3	31.6
Salt	A little				
Colza oil	2.0	0	2.0	0	18.0
Total	672.0	20.4	5.1	220.3	1009.6

of the feeding was about 60 days beginning from September, 1955 and ending in October of the same year. With the completion of the feeding duration, the gall-bladder bile of each dog was extracted, according to the method stated in the previous reports,[2)3)] to measure the bilirubin (total) content.

Results

The results obtained from Group I (the high protein feeding group)

The dogs fed on the high protein food were eight in all; three males and five females. The duration of the feeding was 58–67 days. The bilirubin content in the gall-bladder bile of this group is shown in Table III. The total bilirubin content was 108.0–372.5 mg/d*l*, the mean value being 191.3 mg/d*l*. The gall-bladder bile obtained was generally yellowish dark brown, and showed a viscosity of high degree. This viscosity was most remarkable with Nos. 5 and 9. There was no instance of green or turbid gall-bladder bile.

The results obtained from Group II (the low protein feeding group)

The experimental dogs fed on the low protein food were nine in all; four males and five females. Their weight was 6 to 13 kg., and the feeding duration was 58–67 days. The bilirubin content in the gall-bladder bile of this group is shown in Table III. The total bilirubin content was 40.5–210.0 mg/d*l*, its mean value being 100.1 mg/d*l*.

TABLE III

Bilirubin Content in the Gall-bladder Biles of Dogs Fed on High Protein and Low Protein Food

Group of high protein feeding					Group of low protein feeding				
No. of dog	Sex	Weight (kg.)	Total bilirubin content (mg/d*l*)	Days of feeding	No. of dog	Sex	Weight (kg.)	Total bilirubin content (mg/d*l*)	Days of feeding
1	♀	14	108.0	62	2		8	72.5	60
3	♀	12	124.0	60	4		6	40.5	67
5	♂	10	372.5	58	8		10	98.0	58
7	♀	6	188.0	67	10		12	96.0	58
9	♂	13	256.0	58	12		10	124.0	62
11	♂	12	136.0	62	14		13	210.0	60
13	♀	10	185.5	60	16		6	64.0	62
19	♀	6	160.5	62	18		6	128.0	58
		Mean	191.3		20		10	68.0	60
							Mean	100.1	

The gall-bladder bile obtained from the dogs of low protein feeding group was almost the same in appearance as from the dogs of high protein feeding group, and the difference between the two could be hardly distinguished in color and viscosity. But the comparison of the two results given above showed a marked difference in the bilirubin content in the bile.

In the first group, the mean value of the bilirubin content was 191.3 mg/d*l*. It was much greater than that of second group, which was 100.1 mg/d*l* in its mean. This showed a level of significance less than one per cent in the inductive statistical test. It seems that the difference of the sex and body weight had no noticeable effect on the results obtained.

Summary and Discussion

It has become evident that the bilirubin content in the gall-bladder bile of the dogs fed on a high protein diet for about two months was higher than that of the other group. As regards the difference of the bilirubin content caused by the ingredient of foods given, Toida[4] presented a detailed report. In his experiment, the fluctuation of various components

of the bile of dogs divided into four groups was examined: (1) fed on a high carbohydrate diet; (2) on a high fat diet; (3) on a high protein diet; (4) on a mixed diet. According to his study, a little increase of the mucin and the bile pigment content in the bile was observed with the dogs fed on a high fat diet and on a mixed diet, but no change was observed in the bile pigment content with dogs fed on a high protein diet or on a high carbohydrate diet. Rous and others[5] carried out various experiments, using dogs to examine the relation between carbohydrate and the bile pigment in the bile. They reported that neither a large quantity of carbohydrate content in the food given to dogs nor the additional injection of dextrose increased the total bile pigment content in the bile. The present study clearly proved that both a high protein diet and a low protein diet has a noticeable effect on the bilirubin content in the bile of dogs. It is also known, as Yamada[6] states, that the bile pigment content in the bile of the carnivora is higher than that of the herbivora. In the previous report,[1] the author described that the experiment proved that the bilirubin in the bile of the Caucasian was denser than that in the bile of the Japanese. This leads to the supposition that the Caucasian generally takes more protein or fat than the Japanese. Consequently, the author's conjecture based on his experimental result is that the high protein will also affect the bilirubin content in the bile of the human being.

Conclusion

It has been recognized that the bilirubin content in the bile of the dog fed on a high protein diet was higher than that fed on a low protein diet. From the facts, it has been surmised that with human beings, too, the bilirubin content in the bile may be dependent on the protein content in the food taken.

The writer express his heart-felt gratitude to Dr. Akira Kagaya, Department of Pediatrics, and to Miss Etsuko Satô, nutritionist of this Hospital, for their kind assistance and co-operation given in the present experiment.

References

1) Satô, Published in this Journal as Report II.
2) Satô, Published in this Journal as Report I.
3) Satô, Published in this Journal as Report III.
4) Toida, Mitt. Med. Fak. Kyushu Univ., 1916, **2**, 153.
5) Rous & McMaster, J. Exp. Med., 1923, **37**, 421.
6) Yamada, Igaku-kenkyu (Jap.), 1953, **23**, 10.

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Biochemical Studies on Carbohydrates

CCXIX. Total Synthesis of Lactosamine-octaacetate

By

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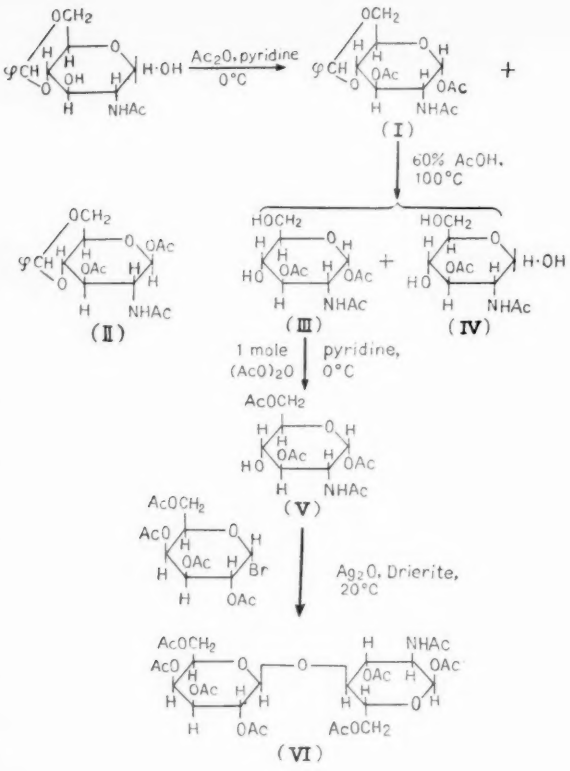
(Received for publication, June 8, 1958)

Yosizawa[1] synthesized N-acetyllactosamine from lactose in 1950, and Kuhn and Kirschenlohr[2] improved the method. The present account deals with a device to totally synthesize the same disaccharide. Since we found that 1,2,3-triacetyl-4,6-benzylidene-α-glucosamine (**I**) is obtainable from N-acetylglucosamine in good yield (1,2,3-Triacetyl-4,6-benzylidene-β-glucosamine (**II**) is yielded much less), it was employed as one of the materials. It was debenzylidenated by heating (20 minutes; 100°C) with 60% acetic acid, and 1,2,3-triacetyl-α-glucosamine (**III**) given was changed into 1,2,3,6-tetraacetyl-α-glucosamine (**V**) by controlled acetylation according to Levene and others[3] and condensed with acetobromogalactose in the manner which Reynolds and Evans[4] applied for preparing gentiobioseoctaacetate from 1,2,3,4-tetraacetyl-glucose and acetobromoglucose. The product (**VI**) (needles) melted at 224°C and had an $[\alpha]_D$ (chloroform) value of +57.7°. No depression of melting point occurred when it was heated with the octaacetate derived from natural N-acetyllactosamine. And its O-deacetylation product gave a golden yellow spot with $R_{lactose}$ of 1.34 as the N-acetyllactosamine did, when paper-chromatographed with solvent butanol-pyridine-water (5:3:2) and sprayed with the benzidine reagent of Raymonds and Morgan[5]. Thus it was identified with the compound in view, although its condensation was very inefficient, yielding only 4% of theory, probably due to steric hindrance.

Experimental

1,2,3-Triacetyl-4,6-benzylidene-α-glucosamine (**I**) and *1,2,3-triacetyl-4,6-benzylidene-β-glucosamine* (**II**). 25 g. of N-acetyl-4,6-benzylidene-glucosamine[6] was dissolved in a mixture of 100 cc. of dry pyridine and 40 cc. of acetic anhydride and stood in a refrigerator (0– −5°C) for 48 hours, the mixture was then poured into 1 *l.* of iced water, and the white precipitate was washed with water and dried. It was extracted with two 100 cc. portions of chloroform, and the solutions were distilled to dryness and recrystallized from abs. ethanol twice. 25.5 g.



(80% of theory) of fine needles was yielded. F.P. 200.5–201.5° (decomp.). $[\alpha]_D^{25}$ (pyridine) $=-100.9°$ (c 0.7). Fehling positive. Soluble in chloroform, pyridine and methanol; slightly soluble in ethanol; insoluble in ether and water. Found: N 3.4; CH_3CO, 31.7. $C_{19}H_{23}O_8N$ requires N, 3.6; CH_3CO, 32.8. As compared with the one below regarding optical rotation, this product is judged as the derivative of α-glucosamine.

The chloroform-insoluble residue above was recrystallized twice from hot abs. methanol. About 2 g. (6.3% of theory) of soft long needles was yielded—the derivative of β-glucosamine. F.P. 248–249° (decomp.). $[\alpha]_D^{25}$ (pyridine) $=-70°$ (c 1.0). Found: N, 3.5; CH_3CO, 32.5. Fehling positive. Soluble in pyridine and hot methanol and ethanol; slightly soluble in chloroform and cold methanol and ethanol; insoluble in ether and water.

1,2,3-*Triacetyl-α-glucosamine* (**III**). Hydrolysis of **I** with 60% acetic acid was found preferable to catalytic hydrogenation of it with Pd-black.

a) Catalytic hydrogenation. 7 g. of 1,2,3-triacetyl-4,6-benzylidene-α-glucosamine was taken up in a mixture of 50 cc. of dioxane and 150 cc. of abs.

ethanol, 3.5 g. of Pd-black was added, and the whole mixture was shaken with hydrogen gas at room temperature. When no more hydrogen was absorbed after 24 hours (The hydrogen gas consumed, 840 cc.), it was brought onto a suction funnel, and the filtrate was distilled. The dry still-residue was extracted with two 50 cc. portions of chloroform, and the solutions were washed with three 100 cc. portions of water. The chloroform-insoluble residue was dissolved in the washings of the chloroformic solutions and distilled to dryness. The glassy yellowish residue (1.5 g.) was recrystallized twice from abs. ethanol by addition of ether. 0.6 g. (11.2% of theory) of crystals in the needle form was yielded. F.P. 142°. $[\alpha]_D^{15}$ (abs. ethanol) $=+123°$ (c 1.0). Fehling positive. Soluble in ethanol, water and hot chloroform; insoluble in ether. It gave a spot with $R_{N\text{-acetylglucosamine}}$ 2.8, when paper-chromatographed (descending) with solvent butanol-ethanol-water (5:1:4, upper layer; 20°C). Found: N, 4.8; CH_3CO, 41.7; OH, 11.78. $C_{12}H_{19}O_8N$ requires N, 4.6; CH_3CO, 42.3; OH, 11.15.

b) Hydrolysis by the aid of 60% acetic acid. 19 g. of 1,2,3-triacetyl-4,6-benzylidene-α-glucosamine was suspended in 200 cc. of 60% acetic acid and refluxed on boiling water, and the clear solution given in 7–10 minutes was heated further on for 20 minutes. When cold, the hydrolysate was washed with two 100 cc. portions of petroleum ether and three 100 cc. portions of chloroform and distilled in vacuo. The syrupy still-residue (light brown; 15 g.) was taken up in about 10 volumes of abs. ethanol, decolorized with charcoal and concentrated to a syrup. It was then dissolved in 50 cc. of abs. ethanol, and after addition of ether to slight cloudiness, placed in a refrigerator. The white crystalline deposit was separated. Yield 12 g. It began to melt at 138° and melted completely at 170°. It gave two spots with $R_{N\text{-acetylglucosamine}}$ values of 2.0 and 2.8 when chromatographed as above, suggesting the di- and tri-acetate. Hence it was dried in an Abderhalden apparatus (P_2O_5, 56.5°C) and extracted continuously with chloroform in a Soxhlet apparatus for 48 hours. The solution was distilled, and the crystalline still-residue was recrystallized twice from abs. ethanol. 8.7 g. (60% of theory) of needles was given. F.P. 142°. $[\alpha]_D^{15}$ (abs. ethanol) $=+125°$ (c 1.0). The product gave a spot at a position corresponding to the triacetate in a chromatogram developed as above, and the mixed melting point showed no depression.

2,3-*Diacetyl-glucosamine* (**IV**). The chloroform insoluble residue above (5 g.) was recrystallized from 50 cc. of a mixture of chloroform and methanol (3:1) three times. 3.6 g. (30.6% of theory) of long needles was obtained. F.P. 173–174°. It mutarotates D-light. $[\alpha]_D^{25}$ (abs. ethanol) $=+32.3°$ (5 min.) $\rightarrow +21.0°$ (45 min.) (c 1.3). Fehling positive. Soluble in water, methanol and hot ethanol; insoluble in chloroform. It showed $R_{N\text{-acetylglucosamine}}$ of 2.0 in a chromatogram developed as above. Found: N, 5.5; CH_3CO, 31.5; OH, 18.8. $C_{10}H_{17}O_7N$ requires N, 5.3; CH_3CO, 32.7; OH, 19.4.

The same diacetate was given in a yield of 92%, when 1,2,3-triacetyl-4,6-benzylidene-β-glucosamine was treated as above. Namely, here almost all the material lost one acetyl besides the benzylidene.

1,2,3,6-*Tetraacetyl-α-glucosamine* (**V**). 5 g. of 1,2,3-triacetyl-α-glucosamine was weighed in the 50 cc. brown flask (*a*) in Fig. 1, dissolved in 25 cc. of dry pyridine and chilled with ice. The solution was vigorously stirred, and 18.5 cc.

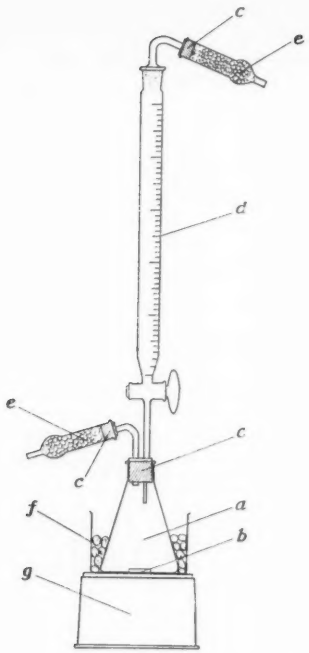


Fig. 1. Apparatus for controlled acetylation. *a*: 50 cc. brown flask. *b*: stirrer, *c*: cork stopper. *d*: 50 cc. burette. *e*: $CaCl_2$ granules. *f*: ice pieces. *g*: rotatory electric magnet.

of a 10% (w/v) acetic anhydride solution in chloroform was added in drops during 1 hour. The mixture was stirred for another 1 hour under continuous cooling and then stood at room temperature for 12 hours. Followingly, it was poured into 100 cc. of iced water, and after standing for several hours, concentrated to a thin syrup (about 40 cc.). The condensate was distilled with toluene in vacuo repeatedly, until it smelled no more of pyridine. The final dry residue was taken up in 50 cc. of water and extracted with 50 cc. of chloroform 6 times in a separatory funnel. The combined chloroformic solutions were distilled to about 50 cc. under reduced pressure and extracted with an equal volume of water many times (10 times). The watery solutions were united, condensed to a glassy solid. Yield 3.5 g. The product here gave two spots with $R_{N\text{-acetylglucosamine}}$ values of 3.2 and 3.4 coinciding with those of 1,2,3,6-tetraacetyl-α-glucosamine and of pentaacetyl-α-glucosamine in a paper chromatogram developed as above. Therefore, it was dissolved in 15 cc. of hot abs. ethanol and placed in the refrigerator for 5 days. The crystalline deposit (long needles), which was the pentaacetate, was eliminated, ether was added to the mother fluid to faint turbidity, and this mixture was placed in the refrigerator

for 1 week, whereby a small precipitate appeared. The supernatant fluid was decanted and concentrated by vacuum distillation. The hygroscopic still-residue amounted to 2.5 g. (45% of theory). $[\alpha]_D^{18}$ (abs. ethanol) $= +87°$ (c 1.01). Fehling positive. Found: N, 3.7; CH_3CO, 50.5; OH, 4.0. $C_{14}H_{21}O_9N$ requires N, 4.0; CH_3CO, 49.6; OH, 4.9.

α-*Lactosamine-octaacetate* (**VI**). A mixture of 2.5 g. of 1,2,3,6-tetraacetyl-α-glucosamine, 2 g. of silver oxide, 10 g. of Drierite[7] and 25 cc. of chloroform was agitated in the 50 cc. brown flask (*a*) in Fig. 1 for 5 hours, and after addition of 0.4 g. of iodine, 20 cc. of chloroform containing 3.2 g. of acetobromogalactose was further added in drops during 1 hour. The flask was taken off the apparatus, closed with a glass stopper (ground) and shaken for 5 days. The reaction mixture was filtered through charcoal, and the filtrate was washed with water thoroughly and distilled in vacuo to dryness. The still-residue was dissolved in 50 cc. of hot moist ether and placed in the refrigerator. The precipitate was recrystallized in a similar manner. 8 g. of crystals thus given was taken up in 15 cc. of chloroform, petroleum ether was added to permanent cloudiness, and the mixture was also stood in the refrigerator. After 1 week, the crystals in needles were subjected to four repetitions of crystallization. The product weighed 0.18 g. (4% of theory). F.P. 224–225°. $[\alpha]_D^{18}$ (chloroform) $= +57.7°$ (c 1.07). It melted at 223–225° when mixed with the octaacetate derived according to Zilliken *et al.*[8] from N-acetyllactosamine which had been separated from the group mucopolysaccharide of pig stomach mucus after Masamune and Yosizawa.[9] The product after deacetylation according to Kuhn *et al.*[10] showed a golden yellow spot with $R_{lactose}$ 1.34 in a descending chromatogram by solvent butanol-pyridine-water (5:3:2) (20°C), when sprayed with the benzidine reagent of Raymonds and Morgan.[5]

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References and Notes

1) Yosizawa, Tohoku J. Exp. Med., 1950, **52**, 145.
2) Kuhn & Kirschenlohr, Ann., 1957, **600**, 135.
3) Raymond, Tipson & Levene, J. Biol. Chem., 1939, **130**, 47.
4) Reynolds & Evans, J. Am. Chem. Soc., 1938, **60**, 2559.
5) Raymonds & Morgan, Naturwissenschaften, 1956, **178**, 1171.
6) Masamune, Okuyama & Sinohara, Tohoku J. Exp. Med., 1958, **68**, 181.
7) Activated by heating at 240°C for 2 hours.
8) Zilliken, Smith, Rose & György, J. Biol. Chem., 1954, **208**, 299.
9) Masamune & Yosizawa, Tohoku J. Exp. Med., 1956, **64**, 267.
10) Kuhn, Baer & Gauhe, Chem. Ber., 1955, **88**, 1713.

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Biochemical Studies on Carbohydrates

CCXX. On Synthesis of N-Acetylallolactosamine

By

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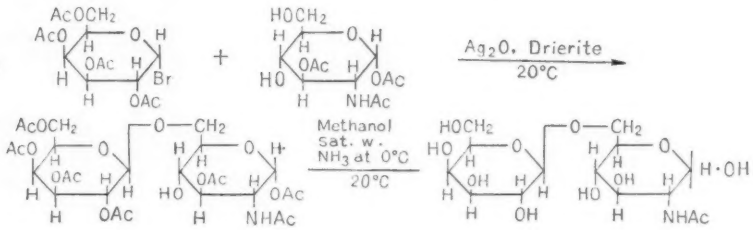
(Received for publication, June 8, 1958)

Kuhn *et al.*[1] synthesized N-acetylallolactosamine by condensation of 1,2,3,4-tetraacetylglucosamine with acetobromogalactose. When we used 1,2,3-triacetyl-α-glucosamine instead of the tetraacetate, N-acetylallolactosamine was produced with about double the yield. This must be because that the steric hindrance in Kuhn's condensation did no occur. To remark, no N-acetyllactosamine formed in our case.



Experimental

N-Acetyl-allolactosamine (**I**). 2 g. of 1,2,3-triacetyl-α-glucosamine[2], 1.5 g. of silver oxide, 6.5 g. of Driertie[3] and 15 cc. of chloroform were mixed in the brown flask in Fig. 1 in the preceding article[2] and stood for 5 hours with occasional agitation. The flask was opened, and 0.3 g. of iodine was added. And quickly thereupon, small portions of 10 cc. chloroform containing 2.7 g. of acetobromogalactose were added through the burette. The flask was taken off the apparatus, closed with a glass stopper (ground) and shaken for 48 hours. The reaction mixture was filtered through charcoal, and the filtrate was washed with water, condensed to dryness and deacetylated according to Kuhn *et al.*[1] The sticky still-residue was dissolved in 50 cc. of water and fractioned with a 5×10 cm. charcoal column by eluting with 5 *l.* of water and 5, 4, 4, 2 and 3 *l.* respectively of 2.5, 5, 7.5, 10 and 12.5% ethanol in succession. The 5% ethanol —10% ethanol eluates were combined and distilled in vacuo as far as possible. 0.85 g. (33% of theory) of a white paste was given. $[\alpha]_D^{18}$ (H_2O) = +27.5°

(c 1.1). It consumed 5.7–5.9 moles (Theory 6 moles) of $NaIO_4$ per mole when oxidized with the reagent in the manner of Masamune *et al.*[4] It showed a brownish red spot with an $R_{lactose}$ value of 1.17 when paper-chromatographed (descending) with solvent butanol-pyridine-water (5:3:2; 20°C) and sprayed with the benzidine reagent of Raymonds and Morgan[5]. Treated with Na_2CO_3 and chromatographed after Kuhn *et al.*[1], it gave a reddish violet spot with an $R_{glucose}$ value of 1.03 or an $R_{lactose}$ value of 2.05 as those authors had observed on their preparation.

The expenses of this work were defrayed by a grant given from the Ministry of Education through the Grant Committee for Scientific Researches. H. Masamune.

References and Notes

1) Kuhn, Baer & Gauhe, Chem. Ber., 1955, **88**, 1713.
2) Okuyama, Tohoku J. Exp. Med., this volume, p. 313.
3) Activated by heating at 240°C for 2 hours.
4) Masamune, Yosizawa & Haga, Tohoku J. Exp. Med., 1956, **64**, 257.
5) Raymonds & Morgan, Naturwissenschaften, 1956, **178**, 1171.

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Chemical Nature of Toxohormone (Nakahara)

Fifth Report

KIK-inactive Toxohormones from Urines of Cancer Patients with Original Tumor in Different Organs*

By

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Formerly,[1] a toxohormone-active substance without KIK-potency was isolated from united urines of cancer patients who were not necessarily the same in original seat of tumor. It was assumed there that the toxohormone active substances from the various urines are identical. Since then, urines of various patients with stomach-, liver-, lung- or mamma-cancer were fractioned separately in the manner applied before, and the products were compared with one another in chemical composition, optical rotation and liver-catalase depression *in vivo*. The results were affirmative for the assumption above, and these results and those previously obtained led the writers to hypothesize that toxohormones, like KIK factors,[2] are produced secondarily in various cells as secretions slightly modified from the normal by a primary principle from the tumor.

Experimental

Preparation of the KIK-inactive Toxohormones from Various Cancerous Urines

Masamune *et al.*[1] were strictly followed. The yields of the products from urines of patients with stomach-, liver-, lung- or mamma-cancer were 61, 56, 46, and 45 mg. respectively per 10 *l.* of urine. They will be referred to as Prep. I, II, III and IV.

Properties of the Preparations

Electrophoresis. All the preparations proved electrophoretically homogeneous in either of solvents veronal-HCl buffer of pH 8.6 and I 0.2 and glycine-HCl buffer of pH 2.0 and I 0.2, as shown in Fig. 1. c 1 ; current 4 mA ; temperature of test 15°C. Mobility :

* 221st report of Masamune and co-workers' "Biochemical Studies on Carbohydrates".

Prep. I, pH 8.6 $\frac{(-0.88)\times 0.00872\times 0.18}{7200\times 0.004} = -4.8\times 10^{-5}$ cm^2·volt^{-1}·sec^{-1}.

pH 2.0 $\frac{(+0.88)\times 0.01692\times 0.18}{7200\times 0.004} = +9.3\times 10^{-5}$ "

Prep. II, pH 8.6 $\frac{(-0.80)\times 0.00872\times 0.18}{7200\times 0.004} = -4.4\times 10^{-5}$ "

pH 2.0 $\frac{(+0.84)\times 0.01692\times 0.18}{7200\times 0.004} = +8.9\times 10^{-5}$ "

Prep. III, pH 8.6 $\frac{(-0.84)\times 0.00872\times 0.18}{7200\times 0.004} = -4.6\times 10^{-5}$ "

pH 2.0 $\frac{(+0.84)\times 0.01692\times 0.18}{7200\times 0.004} = +8.9\times 10^{-5}$ "

Prep. IV, pH 8.6 $\frac{(-0.84)\times 0.00872\times 0.18}{7200\times 0.004} = -4.6\times 10^{-5}$ "

pH 2.0 $\frac{(+0.88)\times 0.01692\times 0.18}{7200\times 0.004} = +9.3\times 10^{-5}$ "

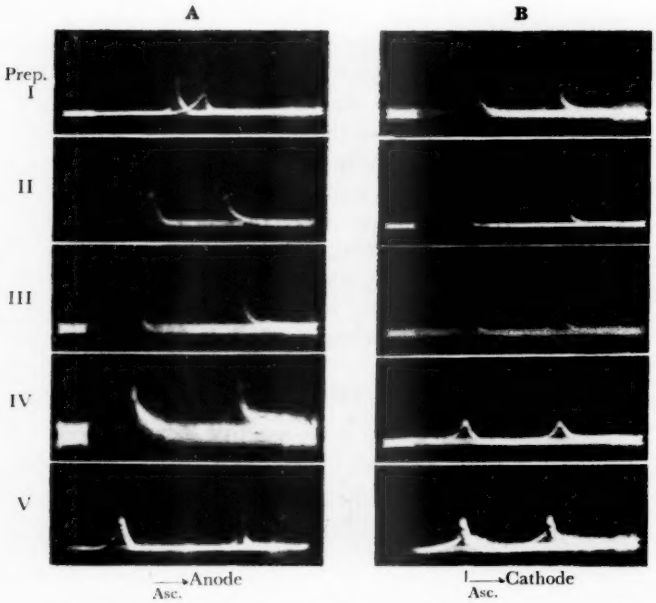

Fig. 1. Electrophoretic patterns of the preparations in the veronal-HCl (**A**) and glycine-HCl (**B**) buffers. The patterns of the normal urine component (Prep. V) corresponding to the cancerous KIK-inactive toxohormones are reproduced from the article of Masamune *et al.*[1]

Liver-catalase depression in vivo. It was measured gasometrically according to Masamune *et al.*[3] The results are detailed in Table I. The toxohormone activities of the preparations were found resembling one

another, 1 mg. of the substances depressing the liver catalase of mice to 60–74%.

TABLE I

Liver-Catalase Depression *in vivo* of the Preparations

Figures express each the average volume of O_2 evolved in per cent of the corresponding O_2 volume in the blank. DCA: depressed catalase activity.

Preparation	No. of test-set	No. of mice used	Amount of substance intraperitoneally injected (mg.)		
			3	1	0.5
I	1	12	62.8	62.1	88.8
	2	9		71.9	85.0
	3	9		70.5	85.0
	DCA			0.68	0.86
II	4	12	59.0	60.1	81.1
	5	9		60.3	91.4
	6	9		73.5	84.0
	DCA			0.65	0.86
III	7	9		64.3	79.7
	8	9		69.4	85.2
	9	9		72.5	89.6
	DCA			0.69	0.85
IV	10	9		63.2	81.8
	11	9		71.1	88.4
	12	9		70.4	85.9
	DCA			0.68	0.86

Composition. The analytical figures obtained are embodied in Table II. They show that all the preparations are of similar composition despite of different seats of original tumor. Furthermore those substances scarcely diverge in composition from the corresponding product from normal urine.

Optical rotation. 0.25% solutions in 0.01 *N* NaOH (pH 9.0) were examined in a 0.5 dm. tube. The specific rotations of the preparations varied from $-111°$ to $-115°$ which is within the limits of experimental error. The corresponding normal toxohormone had $[\alpha]_D^t$ of $-100°$.

Summary and Discussion

It has been revealed that the KIK-inactive toxohormone from can-

TABLE II

Composition of the Preparations

Analytical figures of Prep. V (the corresponding normal secretion) are quoted from the article of Masamune *et al.*[1]

Analysis	In per cent					In equivalents per equivalent weight				
	Prep. I	II	III	IV	V	I	II	III	IV	V
N*	13.2	13.8	13.5	13.8	13.8					
Hexosamine†	2.7	2.6	2.6	2.8	2.9	1.0	1.0	1.0	1.0	1.0
Galactose‡	1.6	1.6	1.8	1.9	1.9	0.59	0.61	0.69	0.67	0.63
Glucose‡	1.0	0.9	0.8	0.8	1.0	0.37	0.35	0.30	0.30	0.33
Mannose‡	2.5	2.6	2.6	2.7	1.4	0.92	0.98	0.99	0.96	0.47
L-Fucose§	0.5	0.5	0.6	0.5	0.5	0.21	0.21	0.25	0.20	0.15
Sialic acid//	3.6	3.5	3.6	3.5	4.4	0.77	0.78	0.80	0.73	0.86
Ash¶	0.8	0.8	0.5	0.4	0.6					

* Micro Kjeldahl. † Masamune and Yosizawa.[6] ‡ Masamune and Sakamoto.[7] § Masamune and Sakamoto.[8] // Werner and Odin (direct Ehrlich)[9]; Calcd. as $C_{11}H_{19}NO_9$. ¶ Pregl method without use of H_2SO_4.

cerous urine does not vary with the seat of original tumor. In conjunction with the previous findings[1),3)-5)] indicating that cancerous toxohormones resemble the corresponding normal components in composition and differ from source to source, the present fact suggests a primary principle elaborated in the tumor which, when transported, disorder the function of various cells to produce cancerous toxohormones as modifications of the corresponding normal secretions. Refer to Kaketa[2)].

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References

1) Masamune, Hakomori, Kaketa, Sinohara & Abe, S., Tohoku J. Exp. Med., this volume, p. 63.

2) Kaketa, *ibid.*, 1958, **67**, 319.

3) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.

4) Masamune, Kaketa & Abe, S., *ibid.*, 1958, **67**, 329.

5) Kawasaki, *ibid.*, 1958, **68**, 119.

6) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.

7) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.

8) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.

9) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.

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Histopathological Differential Diagnosis of Malignant Lymphogranulomatosis (Hodgkin's Disease) from Lymphatic Reticulosarcomatosis

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The opinions upon the histological definition and the essential nature of Hodgkin's disease[1)-3)] are as yet in discord. Especially, these are many problematic points on the histological diagnosis for differentiating this disease from reticulosarcomatosis[4)-7)].

Therefore, the present author of this paper conducted a series of examination of a comparatively large number of materials, with the aim of elucidating the picture of the initial states of these two different diseases so important for their differention and determining the criterion of the-differentiation. The results in summary were as follows.

Experimental

Materials

The tested lymph nodes comprised specimens obtained both at autopsy and clinical examination, consisting of those of 23 cases of Hodgkin's disease (10 autopsied and 13 clinical cases) and 240 reticulosarcoma cases (60 autopsied and 180 clinical cases), all primarily originating in the lymph nodes.

Method

Histological Criteria for Diagnosis and Classification of Hodgkin's Disease and Reticulosarcomatosis

As the histological criteria for diagnosing Hodgkin's disease I adopted the pathohistological characteristics enumerated in the work of Sternberg[3)], in compliance to the general practice. Thus, for diagnostic decision, stress was laid on the detection of Sternberg's giant cells and of granulomata mainly formed by emergence or proliferation of eosinophil cells and reticulum cells, and also, in consideration of the importance of the

initial stage, I examined serially the specimens from the apparent initial stage to the later stages and took specimens containing confirmed transitional forms as bases for reference. The disease types were classified into the three items of paragranulomatous, granulomatous and sarcomatous according to the classification laid down by Jackson and Parker[8].

For the histological diagnosis of reticulosarcoma, I followed the description by Prof. K. Akazaki[7] and adopted the disease types of the undifferentiated, the differentiated and the mixed or transitional types.

Results

1) *Component Cell Groups*

In both the diseases, the star players are the reticuloendothelial cells showing specific reactions and behaviors, so that in the initial stage, always a picture of reticulosis come first into the limelight.

The emergence of Sternberg's giant cells[3] facilitated diagnosis of Hodgkin's disease. In 3 of the tested specimens, such cells were first detected only in the last stage. In the cases where no such cells are detected, however, the structure of the proliferated reticular cells shows peculiarity, for the uniformity in size of their cell bodies in characteristically very marked in Hodgkin's disease and the cells are seen proliferating as if pressing aside the existing numerous lymphocytes. In the initial, no such interrelation between the argyrophil fibres and these reticulum cells, and has been much discussed in the past, could be established.

In the next stage, the nuclei of these reticulum cells become reduced in size and dark staining, and then reticulum cells gain in size of lymphocytes and the general appearance shows a pervading loosences. Their mode of reaction, however, shows a trend of scattered and diffuse proliferation in all the specimens.

Besides, inflammatory cells including eosinophil cells, plasma cells and polymorphonuclear leucocytes are also detected. Eosinophil cells were found in all the cases, and in one case, they were found in groups in the vicinity of the medullary cord and the capsule. The plasma cells were generally scarce in the earliest stage and not detected in 3 cases throughout.

On the other hand, in the lymph nodes of reticulosarcoma, tumor cells show sporadic proliferation in the initial stage, but they are rather uniform in shape and size and soon aggregate into nodules or groups, often presenting a picture closely resembling the so-called "giant follicular lymphoblastoma". In the cases with advanced sarcoma, the original structure of the lymph nodes disappears and comes to be replaced by sarcoma cells proliferated in mosaic patterns. Cell mitosis prospers far more than in Hodgkin's disease. The interrelation between these tumor

cells and the argyrophil fibres is not very close, only a very small number club-like thick fibres being observed.

The appearance of the other types of cells is monotonous here, plasma cells and eosinophil cells being detected on rare occasions only in the initial stage but no polymorphonuclear leucocytes ever coming forth.

2) *Reaction and behavior of blood vessels in the foci*

a) Reaction of the endothelium. The difference in the reactions of the blood vessels in the two diseases should be noted in differential diagnosis.

In Hodgkin's disease, though the findings were not uniform in all the cases, I saw marked swelling and proliferation of the vascular endothelial cells, often completely occluding the lumina of the arterioles and the capillaries and showing the appearance of polymorphonuclear giant cells in formation. No special interrelation, however, was observable between these cells and the argyrophil fibres, such fibres being found only as pre-existing in the vascular walls. In two of the granulomatous type cases, intimal granulomata were found in the veins.

In some of the reticulosarcoma too, hyperplasia of the vascular endothelium was found developed to a considerable extent but such a finding was generally scarce.

b) Changes in an around the walls of the blood vessels. In the focal lymph nodes in Hodgkin's disease, thickening and swelling of the vascular media was notable and collagenous change was severe in the adventitia, which stain red under van-Gieson staining. Often PAS-positive hyaline substances were found deposited on the adventitial fibrous layer. These substances were seen spreading into the stroma too.

In the same parts in reticulosarcomatosis cases, I found a few cases where hyalinization was observable, but the structure of the walls of the blood vessels was usually normal and no deposit of hyaline substance was found in their vicinity.

3) *Cell infiltration beyond the capsule*

In the cases of Hodgkin's disease, cell infiltration beyond the capsules of the lymph nodes was observed in two cases of the sarcomatous type only, in the other cases, marked swelling of the capsules alone being observable, the infiltrating cells consisting only in lymphocytoid small round cells.

In the cases of reticulosarcoma, infiltration of tumor cells beyond the capsules was found meagre in the initial stage, but it increased gradually afterwards and in the later stages, such an infiltration became rather characteristic of the disease.

4) *Fibre formation*

In the initial stage, fibre formation was apparent in both the diseases and a difference in it becomes perceptible only in the advanced stage,

Fig. 1

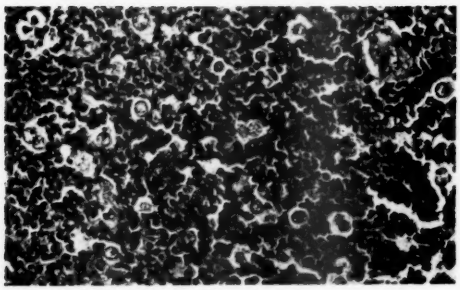

Fig. 2

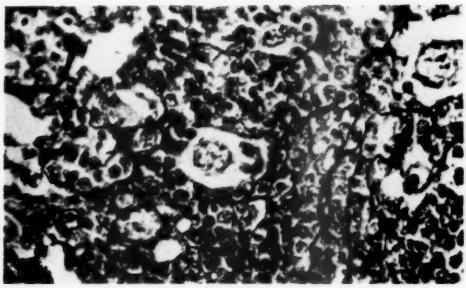

Fig. 3

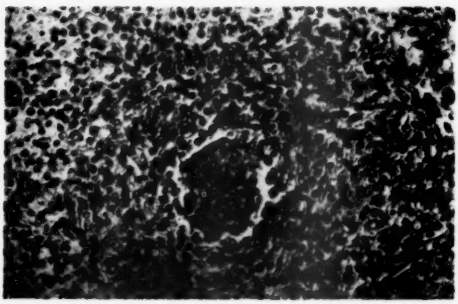

Fig. 1. Hodgkin's disease (Initial stage). Reticulosis is seen and reticulum cells are uniformity in size (H.E.).

Fig. 2. The same case. No interrelation between argyrophil fibers and proliferated reticulum cells (Silver impregnation by PAP).

Fig. 3. Hodgkin's disease. Marked swelling and proliferation of vascular endothelial cells (H.E.).

where in Hodgkin's disease, proliferation and collagenization of argyrophil fibres becomes frank, the response being strong in the stroma, the fibres

Fig. 4

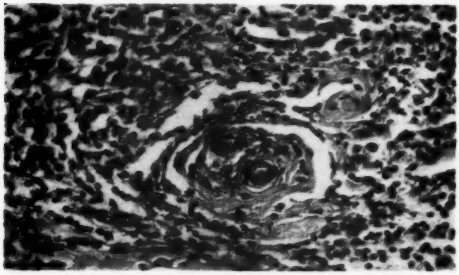

Fig. 5

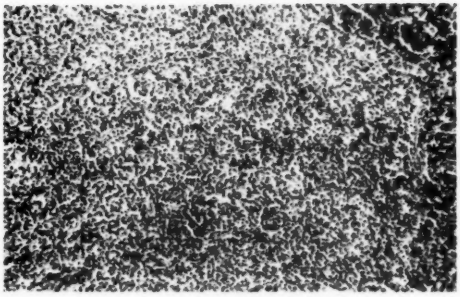

Fig. 6

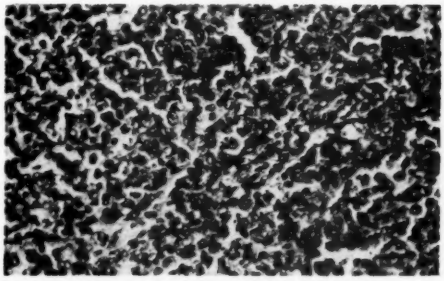

Fig 4. Hodgkin's disease. Fibrosis and collagenous change in perivascular zone (H.E.).

Fig. 5. Hodgkin's disease. Reticulum cells proliferate diffusely (H.E.).

Fig. 6. Reticulosarcoma (Initial stage). Uniformity in size of proliferating reticulum cells (H.E.).

there changing into collagen and forming bundles, while in reticulosarcomatosis, the fibrogeneous changes are not so marked, being entirely absent in the undifferentiated type cases and the proliferation being very poor in the histiocytic type cases ; in some of the reticular type cases, forma-

Fig. 7

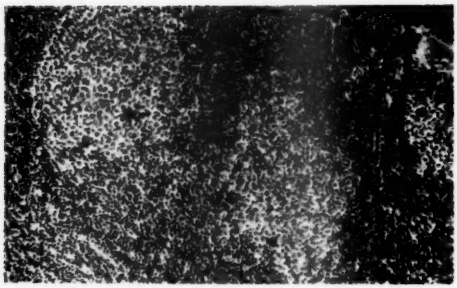

Fig. 8

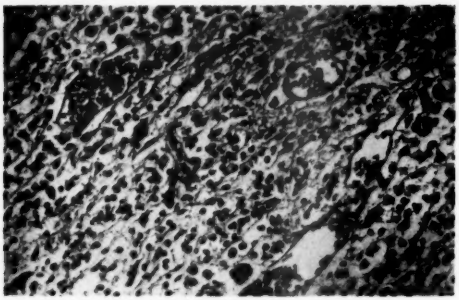

Fig. 9

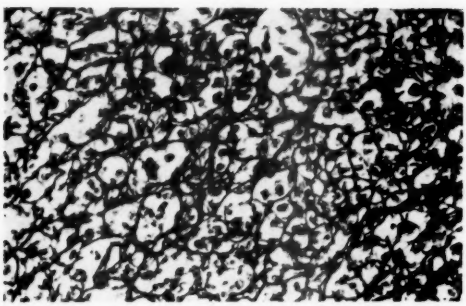

Fig. 7. Reticulosarcoma (Initial stage). Forming small nodules of reticulum cells (H. E.).

Fig. 8. Reticulosarcoma. No findings of vascular changes as in Hodgkin's disease (H.E.).

Fig. 9. Reticulosarcoma (Reticular type). Forming typical net frame work (Silver impregnation by PAP).

tion of argyrophil fibres was indeed peculiar and notable, but in comparison with that in Hodgkin's disease cases, it was still weaker, the stromal

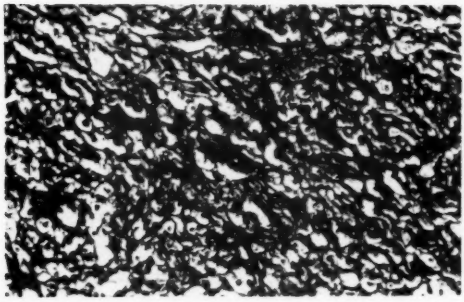

Fig. 10. Hodgkin's disease. Argyrophil fibers and collagenous fibers are more seen than in the case of reticulosarcoma (Silver impregnation by PAP).

parts remaining rather empty, the formed fibres being finer and arranged in typical reticular frameworks.

Discussion

The number of research works treating Hodgkin's disease and lymphatic reticulosarcoma is rather enormous, but comparatively few authors have discussed the morphological difference of these two diseases and those who touched upon the picture of the initial stages of thse diseases are still fewer, only Potter[9], Herbut[10] & Custer[11] being mentionable in this connection.

According to the results of my examinations of the initial stage of Hodgkin's disease a reactive proliferation of reticulum cells, whether in the cortex or in the medulla of the lymph nodes, is a notable item among the histological characteristics of the disease.

Some authors have regarded Hodgkin's disease as a secondary plasmacytomatosis of the lymph nodes, giving prolinence to the plasma cells in their discussion (Maresch[12], Vogt[13], Kusunoki and Frank[14]), and the importance of the presence of eosinophil cells in diagnosing this disease has been already pointed by many authors[7][8][15], but in fact, these cells come into appearance and proliferation rather tardily.

Thus, in diagnosing Hodgkin's disease in its initial stage, detection of the above mentioned cells cannot be relied upon as decisive reasons for falling the diagnosis, but detection of Sternberg's giant cells[3] has been supported by many authors as adequate basis for diagnosing this disease. In my specimens of Hodgkin's disease too, these cells were detected in all the cases, but some authors deny the universal presence of these cells in the disease cases and refuse to recognize the specificity of the cells to the disease (Rössle[16], Robb-Smith[17]). In my tested specimens too, in some of the premortal test-excision specimens regarded as retaining the initial

stage picture, such cells could not be detected, so that we may infer a rather long time elapses before the Sternberg's giant cells[3] make appearance.

Thus, it becomes desirable to obtain some finding adequate in providing a sure ground for dicisive histological diagnosis of this disease prior to the emergence of these giant cells[3].

Roulet[18] has pointed to the polymorphy of the retothelia, the absence of cellular infiltration into the lymph node capsules and the tendency of diffusive proliferation of the Sternberg's giant cells[3] as the outstanding clues to the early diagnosis of the disease.

On the other hand, Kajikawa[19] has reviewed the literature concerning the initial state picture of Hodgkin's disease, and enumerated 1)–3) in the following as its characteristics:

1) Proliferation of cells in the germinal center (Symmers[20], Potter[9], Oliver[21]).
2) Proliferation of lymphoid cells (Barron[22], Reed[15], Longcope[23].
3) Exsudative edematous change (Ziegler[24], Benda[25]).

but considering my results of examinations, I believe an other item should be added to the list, as follows:

4) Reactive proliferation and hyperplasia of the endothelial cells of the blood vessels.

In the following, I will give short comments on these points.

1) *Cell proliferation in the germinal center*

Generally speaking, proliferation in the germinal center is a picture naturally occurring in any inflammatory reactive process and giant follicular lymphoblastoma[26] may be cited as an extremely tumorous type of diseases showing similar pictures.

In Hodgkin's disease, Symmers and other[27] have proved that the hyperplasied follicles strongly suggest the picture of giant follicular lymphoblastoma at first, but soon these follicles are replaced by the cells specific to Hodgkin's disease. In my specimens too, one of those sampled premortally showed hyperplasia of the germinal center suggesting a picture of initial sarcoma, but upon post-mortem examination, a frank Hodgkin's disease was found developed in it.

Potter[9] remarked that his so-called Hodgkin cells proliferate first in the germinal center and soon these cells come to be detected in all the parts of the lymph node. This opinion has been supported also by Moeschlin, Schwartz and Wang[28]. Potter has described these cells as mononuclear cells and many authors have found transition of these cells into Sternberg's giant cells (Coronini[29], von Favre, Croizat[30]). Lennert[31], upon follow-up study, declared these cells to be specific to Hodgkin's disease and to represent the pre-stage to Sternberg's giant cells[3]. The present author has in the above stated that, in the initial stage of this disease, the prolifera-

tion of clear cells pushing to the front as if pressing aside the crowd of lymphocytes is a characteristic feature in the histological picture. These cells sometimes show basophility their protoplasmas and sometimes again the picture of nuclear pycnosis. These cells may correspond to the Hodgkin cells[9] cited above and are always characteristically observable in the earliest stage of inflammatory reactive reticulosis falling under the category of Hodgkin's disease. Such cells may be taken to represent one of the expressive types of reticular cells.

2) *Proliferation of lymphoid cells*

Reed[15], Longcope[23] and Barron[22] have pointed to the proliferation of these cells, but their descriptions are lacking in clarity and not free of objections. Not one of my examined specimens showed any such a finding.

3) *Exsudative edematous change*

Ziegler[24] and Benda[25] have spoken of "ödematöse Durchtränkung" as a symptom in the initial stage of Hodgkin's disease, but they gave no information whether it is due a stagnation of lymph or not.

4) *Reactive hyperplasia of endothelium of blood vessels*

In most of the Hodgkin's disease cases I examined, the endothelium of the minor blood vessels and capillaries was found frankly hyperplasied, but in the reticulosarcoma cases, such a change was very mild, if ever observable. This finding gives a powerful reason for classifying Hodgkin's disease under the heading of inflammatory reactive reticulosis in their wider sense, and serves in differentiating Hodgkin's disease from reticulosarcomatosis in the initial stage already.

There are only a few works[32]-[34] that pay attention to the vascular changes in reticulosarcomatosis, but concerning the changes in the blood vessels, in Hodgkin's disease reports are not rare at all, and in particular, Coronini[29] has given a detailed description. He emphasises the presence intimal granulomata in the veins, which was also confirmed by Kitamura[35] who takes up the intimal granulomata of the venal walls in the traveculae of the spleen and in the lymph nodes as characteristic in Hodgkin's disease, but I could find such granulomata in only two of my specimens.

On the other hand, hyperplasia and proliferation of blood-vessel endothelial cells were frequent and also drastic in all my specimens. Kajikawa[19] also recognized rounding of the endothelial cells, but does not place much importance on this finding. The present author, however, wishes to emphasize the importance of this picture of the proliferation of the endothelial cells in the blood vessel walls as the most outstanding criterion of discriminating Hodgkin's disease from reticulosarcomatosis in the initial stage, seconded only by the detection of Potter's Hodgkin cells. When, in addition, even a few number of plasma cells and eosinophil cells

are found, we may be quite sure in diagnosing Hodgkin's disease.

In the next place, in more advanced stages, the behavior of fibre formation comes into the question; Kajikawa[19] has already reported in detail on this subject. In Hodgkin's disease the fibrous system and the cellular system are said to be in equilibrium and the blood capillaries are important as a constituent of the proliferating fibres, while in reticulosarcomatosis, either the fibrous or the cellular system is said to be predominant. In the results of my studies, some findings endorsing this opinion were found: In the cases of reticular type reticulosarcomatosis, I found typical formation of reticular frame works and genesis of collagenous fibres, but in the undiffentiated type and the histiocytic type cases, fibre formation was far more meagre. In Hodgkin's disease, the argyrophil fibres proliferated with the increase of cell types, the proliferation being more marked than in reticulosarcomatosis.

As described in the above, inflammatory processe comes into prominance high above all the other features characterizing Hodgkin's disease, and it seems quite unreasonable to attribute into a tumor, at least in its initial stage. In this respect, Hodgkin's disease should be essentially distinguished from reticulosarcomatosis.

Conclusion

With the diseased lymph nodes of 23 cases of Hodgkin's disease and 240 cases of reticulosarcomatosis of various types as materials, the author tried to make histological differential diagnosis of these two diseases and arrived at the following conclusions.

1. In the initial stage where the picture of the so-called reticulosis is apparent, the sporadically proliferated reticulum cells lack uniformity of size and in clear cut picture of grouping in Hodgkin's disease, while in reticulosarcomatosis, the cell bodies are comparatively uniform in size and show a tendency of forming groups, giving the general appearance resembling giant follicular lymphoblastoma.

2. The endothelial cells of the small blood vessels are often frankly hyperplasied and proliferated, not rarely stopping up the vascular lumina in the foci of Hodgkin's disease, while no or very slight similar changes are observable in those of reticulosarcomatosis.

3. In Hodgkin's disease, proliferation or hyalinization of collagenous fibres is observed around the focal blood vessels in many cases, but nothing of this sort is observed in reticulosarcomatosis.

4. Proliferation and collagenazation of argyrophil fibers are particularly frank in Hodgkin's disease.

5. Cellular infiltration beyond the capsules of the lymph nodes may be called a characteristic feature of reticulosarcomatosis.

References

1) Hodgkin, T., Med.-Chir. Transact., 1832, **17**, 68.
2) Wilks, S., Guy's Hosp. Rep., 1856, **2**, 103.
3) Sternberg, C., Ergeb. allg. Path., 1936, **30**, 1.
4) Oberling, CH., Bull. Ass. franç. Cancer, 1928, **18**, 259.
5) Rössle, R., Beitr. path. Anat., 1939, **103**, 385.
6) Roulet, F., Virchows Arch., 1930, **277**, 15.
7) Akazaki, K., Byorigaku Zasshi (Japanese), 1943, **2**, 483.
8) Jackson, H. & Parker, F., Hodgkin's disease and allied disorders, New York, 1947.
9) Potter, E. L., Arch. Path., 1935, **19**, 139.
10) Herbut, P. A., Amer. J. Path., 1945, **21**, 233.
11) Custer, R. P. & Bernhard, W. G., Amer. J. Med. Sci., 1948, **216**, 625.
12) Maresch, R., Verh. dtsch. Path. Ges., 1909, **8**, 257.
13) Vogt, E., Frankf. Zschr. Path., 1912, **10**, 129.
14) Kusunoki, M. & Frank, A., Virchows Arch., 1913, **212**, 391.
15) Reed, D. M., Johns Hopkins Hosp. Rep., 1902, **10**, 133.
16) Rössle, R., Cited by Kajikawa (19)).
17) Robb-Smith, A. H., J. Path. Bact., 1938, **47**, 457.
18) Roulet, F., Moderne Probleme d. Pediatrie, 1954, **1**, 706.
19) Kajikawa, K., Juzen-igakkai Zasshi (Japanese), 1956, **58**, 502.
20) Symmers, D., Arch. Path., 1938, **26**, 603.
21) Oliver, J., J. Med. Res., 1913, **29**, 191.
22) Barron, M., Arch. Pat., 1926, **2**, 659.
23) Longcope, W. T., Bull. Ayer Clin. Laborat., 1903, **1**, 4.
24) Ziegler, K., Berliner klin. Wschr., 1911, **43**, 1917.
25) Benda, C., Verh. dtsch. path. Ges., 1904, **7**, 123.
26) Brill, N. E., Baehr, G. & Rosenthal, N., J. Amer. Med. Ass., 1925, **84**, 668.
27) Symmers, D., Arch. Path., 1942, **34**, 385.
28) Moeschlin, S., Schwarz, E. & Wang, H., Schweiz Wschr., 1950, **80**, 1103.
29) Coronini, C., Beitr. path. Anat., 1928, **80**, 405.
30) von Favre, M. & Croizat, P., Cited in Klin. Wschr., 1937, **16**, 607.
31) Lennert, K., Frankf. Zschr. Path., 1953, **64**, 209; 1953, **64**, 343; 1954, **65**, 378.
32) Dudits, A., Zschr. Krebsforsch., 1930, **40**, 229.
33) Roulet, F., Virchows Arch., 1930, **277**, 15.
34) Verhagen, A., Zschr. Krebsforsch., 1940, **50**, 163.
35) Kitamura, S., Fukushima Ishi (Japanese), 1951, **1**, 47.

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Congenital Alveolar Dysplasia as an Important Cause of Death of Premature Newborns

By

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Introduction

The mortality of sucklings and newborns in Japan is tending to fall off in recent years, but still the mortality of premature infants is high and occupy the majority of deaths of newborns. The question why fetus are expelled before maturity is as yet unsolved, and as to the causes of the high mortality there is no adequate explanation to insure general acceptance, despite active researches.

In the statistical figures on the autopsied cases of newborns and premature infants reported by Morison *et al.*[1], anoxia or asphyxia and atelectasis are enumerated as the most outstanding findings beside intracranial hemorrhage, infections and congenital anomalies, indicating how the anomalies in the respiratory function are important as the causes of deaths of newborns.

Now, as one of the sine qua nons for a fetus to maintain life and to grow outside the mother's womb, we must reckon with its ability of apssing over from placental respiration to pulmonary respiration. In premature newborns, however, the respiratory function of the lungs is as yet immature and this frequently leads to anoxia, as has been often emphasised, and many researchers have inferred that such a respiratory hypofunction threaten the life of newborns. We can enumerate a number of factors that induce such anoxia, and in particular, Potter's[2] study on the formation of hyaline membrane has called attention of many researchers in this connection. He has reported that the deaths due to formation of hyaline membrane lining the alveolar walls account for 40.5% of the total mortality of premature newborns. Mac Mahon,[3] however, placed more importance on his congenital alveolar dysplasia and more recently Kaufman[4] also supported his opinion. Some point to pneumonia as the most frequent cause of deaths of premature infants, and we have as yet no final agreement on the cause of anoxia and consequently, the cause of deaths

of anoxia of premature infant in sight. Therefore, the present author, with the aim of contributing to the elucidation of the problem, studied the pulmonary tissue of premature newborns with that of normal fetus and mature newborns in comparison and obtained interesting findings, as reported in the following.

Experimental

Subjects and Method

The subjects of my study consisted in 74 cases in total of fetus and newborns autopsied at this Pathological Laboratory, Tohoku University School of medicine, in these 7 years, comprising 52 cases of so-called premature infants below 2,500 g. in weight at birth and control cases of 8 full-term-born infants after 10 months of normal pregnancy and 14 still-births infants delivered in the pregnancy months of the 5th to the 10th. The premature cases included 19 of 8 months, 23 of 9 months and 10 of 10 months, or 11 cases of 1,000–1,500 g. at birth, 24 of 1,501–2,000 g. and 17 of 2,001–2,500 g.. Of these 4 cases lived less than 24 hours after birth, 7 for 25–48 hours, 12 for 3–5 days, 17 for 6–10 days, 11 for 11–20 days and 1 for 21–30 days.

Anomalies at birth consisted in 14 cases of toxemia of pregnancy, 15 cases of twins, 3 cases of placenta praevia, 3 cases of early rupture of water bag, 1 case of placenta praevia, 3 cases of early rupture of water bag, 1 case of hydramnion and 2 cases of contracted pelvis. In 27 cases no clinical anomaly was perceptible.

The histological preparations were made by fixing the materials in 10% formalin solution, cutting into paraffin embedded and staining with hematoxylin and eosin, van Gieson, Mallory's trichrome stain and Pap's silver impregnation, while sudan III staining was applied to the frozen sections.

Histological Findings of the Lungs

1) Lungs of the still-born fetus

Lungs of 5 months of pregnancy

At this stage, the alveolar septa are considerably narrowed down but the lung is as yet in a state of low differentiation. In general, the picture of infantile fetal mesenchyma is still observable. Stellate of fusiform cells are found in comparatively sporadic existence and fine fibres are found among them. The bronchi and the bronchioli are markedly developed and proliferated at this stage. The differentiation of the blood capillaries in the alveolar septa however, is as yet not advanced, being covered by loose stroma, some of them lying in direct contact with the internal alveolar walls and some embedded in the thick stromal tissue of the alveolar septum between its basal membranes. The alveolar epithelium consists mainly of cylindrical or sometimes of cuboidal cells in close epithelial combination. The alveolar walls and the alveolar septa are considerably narrowed, as stated above, but are as yet thick, under silver staining, argyrophil fibers are seen forming loose net-works, but the fibres are still weak staining.

Lungs of 6 months of pregnancy

The stromal tissue of the lung has thinned down the more and the cells there show a perceptibly better differentiation than the same in 5 month fetus. The stellate cells have changed into round cells and the alveoli, have amplified both in number and size. The alveolar epithelium of cuboidal cells have gradually flattened, some of them having decidnated into the alveolar space and newly formed and rapidly developed blood capillaries have run into the alveolar septa. The alveolar walls are slightly edematous and their basal membranes are found formed by argyrophil fibres, but they are as yet in the form of loose-knit baskets, wherein numerous finely branched argyrophil fibres are found in complex intertwining.

Lungs of 7 months of pregnancy

The stromal tissue of the lung has markedly decreased in quantity from that of 6 month fetus and filled up by dense, differentiated and round cells. The alveolar epithelium is further flattened and decidnated in more numerous places, where blood capillaries covered by very thin fibrous membranes are found exposed. The alveolar walls have further losed in thickness, the basal membranes are conspicuous in formation, and, in particular, it is notable that the argyrophil fibres in the connective tissue around the bronchi and the blood vessels which consisted in fine intertwining fibres in the prior month have now fused into a few stout fibres.

Lungs of 8 months of pregnancy

In the fetus of 8 months, the alveolar walls have become very thin, consisting of only 1–3 layers of mature cells, the basal membrane in the alveolar walls are markedly well developed and the number of exposed blood capillaries have steeply risen in number. The alveoli increase in number, size and curvature, so that the picture approximates that in mature newborns. The alveolar epithelial cells are flattened and reduced in number.

Lungs of 9 months of pregnancy

The alveoli rise in density and individually more dilatated, their epithelial cells extremely flattened and decreased and the exposure of blood capillaries shows little difference from that of mature infants. The alveolar walls are reduced to the thinness of that of mature newborns, consisting of 1–2 layers of cells. The basal layers are well developed and the alveolar septa are uniformly thin, the argyrophil fibres being arranged in 1–2 rail-like lines instead of the loose-knit baskets in the earlier stage and some of them being noted running thence into the alveolar epithelial layer in chains of circular rings.

Lungs of 10 months of pregnancy

The findings here are essentially similar to those in 9 month fetus, only the differentiation being more advanced. The alveoli are uniformly dilatated as in mature newborns, their walls more thinned down and edema and cellular infiltration are less perceptible than in the preceding stage. The blood capillaries are still hyperemic and are nearly completely exposed on the inner surface of the alveoli. Argyrophil fibres are well developed, not only in the stroma but also in

the pleura and the interlobular fibrous layers, have become thicker and more densely and compactly arranged, showing the appearance of as if keeping pace with the development of the lung.

2) Lungs of premature newborns

The lungs of premature newborns, it may be noted, are macroscopically stiffer and poor in air content in many cases, giving the impression of splenization observed in pneumonia cases. But upon histological examination, we find, instead of the picture of pneumonia, a very characteristic picture, namely, the alveolar walls are markedly thickened and the Mac Mahon's[3] so-called congenital alveolar dysplasia becomes apparent. Of these specimens, I estimated the individual level of differentiation by the thickness of the alveolar walls and septa, the development of the argyrophil fibres, the extent of exposure of blood capillaries in the alveolar cavities and the differentiation of the cells, then determined the extent of differentiation in the foci and the highest level of possible differentiation in these cases and finally decided the three following types for classifying the dysplasia by the mode of focal formation; (1) diffuse type, (2) localized type and (3) mixed type. By $+\alpha$ is indicated that though the formation of the alveolar walls and septa and the blood capillaries by the argyrophil fibres corresponds that of a normal fetus of the indicated months of pregnancy, there is some slight development observable, namely, the individual fibres have larger diameters or the alveolar cavities are better deployed.

In making estimation of the histological pictures of the individual cases, the above-mentioned standards for classification have been followed, and in particular, the thickness of the alveolar walls was composed with that of the well-differentiated alveolar walls of mature infants and the cases were divided into the grades ##, #, +, ± and − in this respect.

Summary of findings of the lungs of 19 premature infants of 8 months of pregnancy

Generally speaking, the alveolar walls were found markedly worse-developed than in the controls of the same monthly age, in some cases, the lowest level of differentiation not higher than that in 6 month fetus. The stromal tissue is abnormally abundant, preventing the expansion of the alveoli, which are lined by cylindrical epithelia without gap and are usually subnormal in number. The alveolar walls are thick and composed of several layers of undifferentiated cells, the basal layers are ill developed and the argyrophil fibres form loose basket-works. The stroma around the blood capillaries and the bronchi is thick and loose and consists of young weakly silver-staining fibres, which are the finer, the more the immature, that is, the more hypoplasied they are but usually thicker, the less severe the hypoplasia. The thickness of the argyrophil fibres is correlated with the number of the days the subject survived after birth, so that even in the cases where the hypoplasia was severe (the stromal tissue was thick as a whole and the fibres rich in branches), I could find my so-called $+\alpha$ findings more often as the daily age of the newborn rose. Such $+\alpha$ findings may be looked upon as due to post-natal developments.

Pneumonia was found in 26 cases, including mild cases, and in particular, suppurative pneumonia of specific form was observed in the cases that survived only a small number of days, but in no case could I found formation of pulmonary hyaline membrane so noted with emphasis by Potter[2]. In summary of the above, I may say that the differention apparent in the lung foci was of the level corresponding to that in 6 month fetus at the lowest and that in 9 month fetus at the highest. By classification types, localized and diffuse types were the more prevalent in the cases with short lives after birth and the more the subject had survived, the mixed type became the more frequent.

Ditto of 23 premature infants of 9 months of pregnancy

At this stage, the level of alveolar dysplasia was estimated at (#) in 2 cases, at (++) in 12 cases, at (+) in 5 cases, at (±) in 3 cases and at (−) in 1 case, suggesting that the dysplasia generally falls in severity as the months of pregnancy grow older. The alveolar walls of the subjects, however, were found still thicker than the same of the control cases, so that the blood capillaries that should have been completely exposed by this time are found often yet unexposed. In a normal fetus of this month of pregnancy, the argyrophil fibres are already well developed, the basal membrane are formed by thick rail-form fibres and argyrophil fibres are found running into the alveolar epithelium forming round-linked chains, but in my specimens of premature infants such findings were absent and the development of the argyrophil fibres was of the order of 7 month fetus, such fibres often running in loose, basket-wise arrangement. The average level of focal differentiation was that of 7–8 month fetus and the highest of 9 month fetus. Of the alveolar content, hyaline membrane formation was notable in 3 cases. This membrane lining the interior alveolar wall stains dark under eosin, not at all or very light under sudan III and blue under Mallory's method. Cases of pneumonia were rarer, only 2 cases of desquamative pneumonia having been observed including one in whom the above hyaline membrane formation was observed.

A similar correlation between the types of anomalies and the daily age of the subject was found here as in the preceding, namely, the diffuse type was prevalent in the cases that survived less than five days after birth but the mixed type was more frequent among those who survived longer.

Ditto of premature newborns of 10 months of pregnancy

When the subject had reached this age before birth, the development of the lung was found to have advanced considerably beyond the level reached in the preceding. So, no particularly severe case of dysplasia could be found, only one case of (++) grade being observed and 3 cases showed scarcely any trase of hypoplasia. But atelectasis was partially found still serious in some cases and slightly too thick alveolar walls suffering from mild edema and cellular infiltration were not absent either. In such parts, the argyrophil fibres were found to form frail but coarse-meshed net-works as found normally in 8 month fetus, but in well-developed parts, the differentiation reached the level of 10 months of normal pregnancy. In the latter, well-developed rail-form basal membranes lined the alveolar septa, argyrophil fibres run into the alveolar epithelium and formed

chains of rings together with other argyrophil fibres running around the blood capillaries. Pneumonia was rather frequent at this stage, being diagn in 5 cases, but no case of hyaline membrane formation was observed.

The level of differentiation in these cases thus falls to that of normal fetus between 8 and 9 months at the lowest and that of 9 to 10 month fetus at the highest. The correlation between the types of dysplasia and the daily age of the subject was not so clear as in the preceding stages.

3) Ditto of 8 mature newborns

The subjects comprised full-term infants delivered after normal pregnancy of 10 months, between 2,500 g. and 3,380 g. in weight at birth, mature both in weight and height. Usually, the alveoli were found very densely arranged and fully dilatated, but in 2 cases, moderate atelectasis was observed. The alveolar walls were moderately thickened but scarcely any edema or cellular infiltration was apparent. The argyrophil fibres were well developed, stout, forming rail-form basal membranes, and running into the alveolar epithelium, they formed chains of rings in company of similar fibres in the walls of the exposed congested capillaries, no case was found suffering from hyaline membrane formation, but 3 cases of pneumonia were observed. The level of focal differentiation was that of 8 month fetus $+\alpha$ at the lowest and of 10 month fetus at the highest. Almost all the cases showed the mixed type dysplasia which, in summary, were of the grades (++) in 1 case, (+) in 3 cases and (−) in the 4 remaining cases.

Summary and Discussion

In the above, I have outlined the results of my histological study of the lungs of 52 premature newborns, with the lungs of 14 fetus of more than 5 months of pregnancy and 8 full-term newborns as control. In particular, I laid stress on the progress in the differentiation of the alveolar septa and the stroma, the teleclasis and the function of the alveoli in the later fetal stage. There are not a few works published on the histological study of growth of human fetus, for example, by Akazaki[5], Hosaka[6], Cooper[7] and Clara[8]. The fetal lung markedly advances in its progress at the end of the 7th month and during the 8th month of pregnancy; the alveolar walls grow thinner, the stroma decreases in quantity, the alveolar epithelium grows further flattende, the blood capillaries become more exposed in the alveoli, which increase further in number, size and the number of their folds. Such findings reflect that the pulmonary alveoli have become sufficiently expansible at this stage and that under adequate nursing, pulmonary ventilation may be successfully effected. In the 9th month, the alveolar walls become uniformly ever thinner, rail-form basal membranes come to be formed and thence fibres run toward the alveolar epithelium in the form of chains of rings. In the 10th month, the development of the lung is as complete as in full-term newborns.

In the lung of premature newborns, however, I found in many cases that the alveolar walls are thicker than in normal fetus of the same month, the blood capillaries are covered by loose connective tissue, preventing adequate exposure of the capillaries and showing the picture of Mac Mahon's[3] so-called congenital alveolar dysplasia.

Thus, in the newborns of 8 months of pregnancy, the alveolar walls are composed of yet undiffisentiated cells, the capillaries are not sufficiently exposed and the argyrophil fibres are fine and arranged in loose-knit net-on basket-form. The stroma and the interlobular fibres show parallel states of low differentiation. In premature newborns of later birth too, scarcely any case showed the same level of differentiation as the fetus of the same months, but the argyrophil fibres were often grown thick, in spite of the general dysplasia. This thickening of the argyrophil fibres to the level of normal fetus of the same months despite general low differentiation is a finding to be specially noted. For if the argyrophil fibres become thick while the alveolar walls are comparatively thick, the expansion of the alveoli is hindered and consequently the respiratory power after birth is limited, resulting in a picture of persisting dysplasia. Such a dysplasia of the lung tissue is often observed locally, and with the increase of the days of survival the localized and mixed types of lung dysplasia tend to become more prevalent. Such a dysplasia of the alveoli is sometimes observed in company with general dysplasia of the individual but sometimes also quite independent of general bodily growth, especially in the cases of births in the 8th or the 9th month of pregnancy. The frequency of alveolar dysplasia is not correlated with the existence of anomalies in the maternal body, but the younger the mother, and also the lower the number of months of pregnancy, the frequency of dysplasia was found the higher.

A cause of deaths of premature infants looked upon as important is pneumonia. As stated above, I found 11 cases of comparatively serious and 2 of mild pneumonia, but little correlation was perceptible between the frequency of pneumonia and that of alveolar dysplasia. But I can point out that the types of pneumonia diagnosed in the cases with alveolar dysplasia were limited to the hemorrhagic and the desquamative, no suppurative focal type pneumonia developed from the common badly exudative type being ever found among them. This peculiarity may be deemed to be related with the incrassation of the alveolar walls and the insufficient exposure of the blood capillaries. Besides, such pneumonia in premature newborns make appearance soon after birth in complication with alveolar dysplasia.

Studies on the hyaline membranes in the lungs of new-borns have been reported on by Farber & Wilson,[9] Gavallér[10], Reutter[11], Potter and others[2], and particularly Potter *et al.*[2] have asserted that such membranes

come forth very frequently in premature newborns and have a high significance as a cause of their deaths. Among my 52 tested cases, however, I found only 3 cases with hyaline membranes in formation. Hyaline membranes, at least typical ones, are hardly overlooked by anyone, and this discrepancy of my findings from those of Potter *et al.*[2] is not easy to explain, but we may surmise that when we take the trouble to search for even the slightest traces of hyaline membranes the frequency might be indeed surpassingly ligh. We may, however, feel justified in doubting whether such tiny formations as are basely detectable under a microscope could actually have such a serious effect upon the pulmonary ventilation of a premature newborn so as to threaten his life. The genesis of hyaline membranes, on the other hand, was formerly attributed to aspiration of amnion or oxygen intoxication, but more recently, anoxia or disturbed permeability of the capillary walls has come to be regarded as the most outstanding factor and the opinion citing dysplasia of the pulmonary capillaries or consequently of the entire lung, or of both the lungs and the heart as the cause of hyaline formation has come to the fore. Accordingly, the formation of hyaline membranes may be considered as resulting from pulmonary dysplasia in the case of premature newborns, and if we admit that the membranes may directly cause death of such cases, their frequency is not at all high, as MacMahon's and my researches have revealed. We should rather take up the pulmonary hypoplasia that causes the formation of such hyaline membrane as the first cause of deaths of premature infants.

In conclusion, as a clinical note, I may point out that alveolar dysplasia is frequently observable in the cases that suffered from asphyxia or cyanosis at birth.

Conclusion

1. In a great majority of dead premature newborns I examined, the alveolar walls were found thicker than the counterparts in normal fetus of the same month of pregnancy and the exposure of blood capillaries inside the alveoli was insufficient. These capillaries being covered by coarse well-remified agryrophil fibres. That is, the picture of the so-called congenital alveolar dysplasia was apparent.

2. While the entire construction by argyrophil fibres remains in the state of low differentiation, the formative fibres gain in diameter as the months of pregnancy grow and this phenomenon is enhanced further with the increase of the number of the days of survival of the infant after birth.

3. In the case when the differentiation of the lung structure has kept abreast of the fetal age through 8 months or more, in principle, the newborn should be able to enjoy adequate respiratory function ex utero but

in the case of premature newborns, even when delivered after more than 8 months of gestation, if the differentiation of its lung tissues is in arrear of that of normal fetus of the same age, only the size of the argyrophil fibres is often found similar to that in normally grown fetus, so that we can infer the repiratory function will be further impeded after birth.

Fig. 1

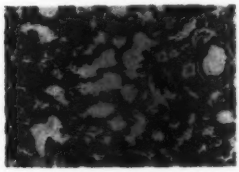

Fig. 2

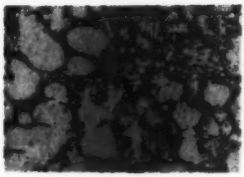

Fig. 3

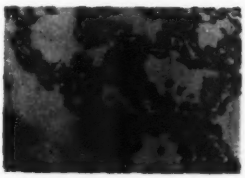

Fig. 1. Alveolar dysplasia, diffuse type (Premature infant born in 8th month of pregnancy and lived 2 days after birth). The alveolar walls uniformly thickened and edematous, retaining traces of infantile mesodermal tissues. Exposure of capillaries incomplete. Differentiation level approximately that of normal 6 month fetus. (H.-E. stained, low magnification).

Fig. 2. Alveolar dyspasia, localized type (9 month premature infant, lived 7 day after birth). A focus of ill-differentiated alveolar walls in the right half of the picture, where the walls are edematous and the exposure of the capillaries are not adequately advanced. A better-differentiated part nearly risen to the level of the corresponding fetal age is visible to the left. (H.-E. stained, low magnification).

Fig. 3. Alveolar dysplasia (8 months premature infant, lived 2 days after birth). Lowest differentiation of the level of 6–7 month fetus in findings of lung. (Pap's silver impregnation, low magnification).

4. Cases of severe alveolar dysplasia are found the more frequent, the younger the intrauterine age of the premature newborns and the lower

their bodily growth at birth.

5. No special correlation was found between the frequency of pulmonary dysplasia of premature newborns and anomalies during pregnancy. Toxemia of pregnancy, however, is the most frequent among the anomalies observed during gestation before premature delivery, and in such cases, hydropsia was frequent both in the alveoli and the alveolar walls of the newborns.

6. The frequency of alveolar dysplasia is the higher, the lower the age of the mother below 29 years.

7. Pneumonia was found in 13 cases, including mild cases, and the type of pneumonia was found closely correlated with the severity of the alveolar dysplasia and the day number the newborn survived after birth. Namely, pneumonia in premature infants often occurs soon after birth in complication with alveolar dysplasia, always showing reaction by desquamation of the epithelium or heavy hemorrhage, but never showing the form of common focal pneumonia.

8. Cases showing formation of hyaline membranes were very rare, i.e., only 3 among my 52 subjects. I have discussed the relation between the genetic cause of these membranes and alveolar dysplasia.

9. Alveolar dysplasia may be observed in the lung of full-term newborns too, but here the anomaly is generally mild in grade.

10. Alveolar dysplasia is particularly frequent in newborns that suffered from asphyxia and cyanosis at birth.

References

1) Morison, Fetal and neonatal pathology, Butterworth, London, 1952.

2) Potter, Pathology of the fetus and the newborn. The Year Book Publishers, Inc., Chicago, 1952.

3) Mac Mahon, Amer. J. Path., 1948, **24**, 959.

4) Kaufman & Spiro, Arch. Path., 1951, **51**, 434.

5) Akazaki, Nisshin-Igaku (Jap.), 1943, **32**, 930.

6) Hosaka, Hokuetsu-Ikaishi (Jap.), 1942, **57**, 1112.

7) Cooper, J. Path. Bact., 1938, **47**, 105.

8) Clara, Zschr. mikrosk.-anat. Forsch., 1936, **40**, 147.

9) Farber & Wilson, Amer. J. Dis. Child., 1933, **46**, 572, 590; Arch. Path., 1932, **14**, 437, 450.

10) Gavallér, Verh. dtsch. Ges. Path., 1956, **40**, 191.

11) Reutter, Gyne., 1954, **137**, 367.

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Electromyographic Studies of the Abdominal Wall Muscles in Visceroptosis

I. Analysis of Patterns of Activity of the Abdominal Wall Muscles in Normal Adults

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In recent years electromyographic studies of the abdominal wall muscles have been made from many points of view such as in, respiration[1)-4)], pregnancy[5)], défense musculaire[6)-8)] and also in cases[9)] where sexual hormones have been injected. However, there could be found no literature treating the role of the abdominal wall muscles in visceroptosis. Floyd and Silver[10)] (1950) established an electromyographic kinesiology of the abdominal wall muscles of the normal adults by using surface electrodes. The scope of the present study was to determine the condition of the abdominal wall muscles in visceroptosis according to the method of the authors mentioned above.

In this paper, the results are given on the observations on the condition of the abdominal wall muscles in normal adults which were used as a control for the study of the cases of visceroptosis.

Experimental

Method

The technique employed was essentially the same as that described by Floyd and Silver (1950). The subjects employed in this investigation were 15 healthy addults, 10 males and five females, ranging in age from 17 to 40 years. All had relatively thin fat layers on their abdominal walls, and obese subjects were strictly avoided. An eight channel electroencephalograph ME-3D with ink-writer oscillographs manufactured by the Nihon Koden Company was used. Eight electromyograms were recorded simultaneously from different muscles, viz. the internal and external oblique, and the rectus abdominis of both sides. The electrical lead employed was the same as that used by Floyd and Silver. Surface electrodes were used in preference to needle electrodes for the following reasons:

1) A large volume of muscle can be sampled ; thus a better indication of the bulk participation of the muscle is gained, and the recording of unrepresentative motor units is less likely.

2) Surface electrodes have a great advantage since they give the subject less apprehension, inflicting no pain and make it possible to tie the abdominal bandage over the electrodes. Thus, the silver-silver chloride surface electrodes of 1.0 cm. in diameter were used in all experiments. It was not necessary to use Braun tube oscillographic recordings for this type of study, because details of waveform were of no significance. The main features were amplitude and frequency, which were adequately recorded with the ink-writers. The time constant was 0.1 second. All experiments were performed on an insulated couch in a shielded room to protect the waveform against interference by alterating current.

Electrode lay out : First, the skin of the anterior abdominal walls of the subjects were generally wiped and cleaned with alcohol gauze so that the electrode resistance might be less than 5–10 kΩ. Pairs of electrodes were coated with electrode jelly, placed symmetrically on each side of the mid-line over the abdomen in the positions stated below and finally fixed to the skin with adhesive patches. The earth connection was made with an electrode placed approximately in the midline above the symphysis pubis.

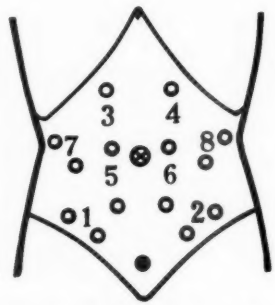

Fig. 1. 1 : r. internal oblique ; 2 : l. internal oblique ; 3 : upper part of r. rectus abdominis ; 4 : upper part of l. rectus abdominis ; 5 : lower part of r. rectus abdominis ; 6 : lower part of l. rectus abdominis ; 7 : r. external oblique ; 8 : l. external oblique. • : Earth connection.
These numbers are applicable to all figures in this series.

1) On the internal oblique (1, 2 in Fig. 1)

A pair of electrodes was placed on each side over the triangle bounded by the lateral edge of the rectus sheath, the inguinal ligament and the line joining the spina ilica ventralis to the umbilicus. In this area the fibres

of the internal oblique are separated from the skin only by the external oblique aponeurosis, and hence are accessible for study with surface electrodes as described by Floyd and Silver. The electrodes were placed in a line parallel with the inguinal ligament, i.e. over those fibres which are over the inguinal canal and are inserted into the conjoint tendon.

It is possible that the internal oblique electrodes may pick up from the lowest fibres of the M. transversus abdominis. These fibres also arch over the inguinal canal and are conjoined with the internal oblique fibres. The similarity of origin, course, and insertion between these two sets of transversus and internal oblique fibres makes it likely that their function is also similar. For this reason no attempt is made to differentiate between them.

2) *On the rectus abdominis* (*3, 4, 5, 6 in Fig. 1*)

The M. rectus abdominis was divided into the upper half and the lower half. Namely, one electrode was placed over the upper part of the rectus abdominis between the Xiphisternum and the umbilicus at an equal distance from the mid-line. The other was placed at the height of the umbilicus. This pair of electrodes was used for testing the upper half though the latter one was also used for testing the lower half. The other one for the lower half was placed over the lower part of the rectus abdominis between the umbilicus and the symphysis pubis. Every pair of electrodes was placed longitudinally in the line parallel with the fibres of the rectus abdominis. Care was taken to avoid the tendinous intersections in each case, though very little difference in the record was seen when an electrode was placed over an intersection.

3) *On the external oblique* (*7, 8 in Fig. 1*)

A pair of electrodes was placed 3.5 cm. apart on each flank, above the anterior half of the crista ilica. The posterior electrode was placed higher than the anterior so that the axis of the pair was oblique extending downwards and forewards in the line of the muscle fibres.

Results

When the subjects are quiet in a supine posture on the insulated couch, no action potentials are recorded from any muscles. This is illustrated on the left side of Fig. 2. But in some nervous subjects a slight discharge is indicated from the obliques even in a resting state. Nervous subjects do not relax completely. The emotional or psychological state of the subjects seemed to be reflected in the degree of resting discharge. The electrocardiograms (E.C.G.) superimposed on most records were seen in this experiment (Fig. 2). But the electromyograms (E.M.G.) and E.C.G. records are easily distinguishable ; the presence of the E.C.G. does not confuse the interpretation of the E.M.G. record.

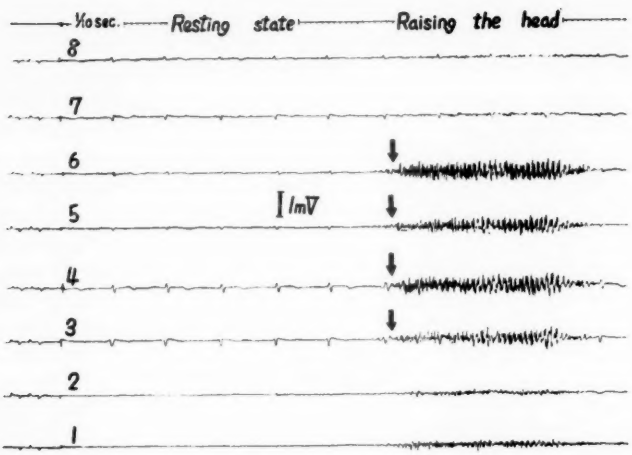

Fig. 2. a) The left half shows a resting state when the subject supine and quiet. No action potentials are recorded from any muscles. b) The right half shows the muscle-action potentials demonstrating the pattern of activity for raising the head. The E.C.G. can be seen in the recti and external obliques.

The right half of Fig. 2 shows the pattern of activity for the movement of head lifting i.e. raising the head off the couch by flexion of the neck. This movement is carried out against gravity. Strong discharges are picked up from the rectus muscles. These discharges are characterized by an amplitude of 500–1000μV. Discharges of the oblique muscles are very slight. The significance of this phenomenon is that the recti are mainly involved and the oblique muscles play a very minor role in this movement. The recti may be, therefore, regarded either as an agent in a general flexion movement, involving the neck and trunk, or as a fixator of the thorax.

When the activity of the corresponding muscles of both sides is compared, a small difference is commonly seen. This difference may be due to functional asymmetry. The movement executed by the subjects may not always be symmetrical, and this asymmetry may be reflected in the difference in activity recorded from the two sides.

When the left or right leg is raised, the action potentials are recorded from the ipsilateral internal obliques, as shown in Fig. 3. When both legs are raised and the pelvis is fixed by the trunk musculature, all abdominal muscles are in action, and the contractions are especially remarkable in the recti and the external obliques (Fig. 4).

When the intra-abdominal pressure is raised by means of the stoppage of breathing and the lowering of the diaphragm, the action potentials from the internal and external obliques are mainly recorded and the internal

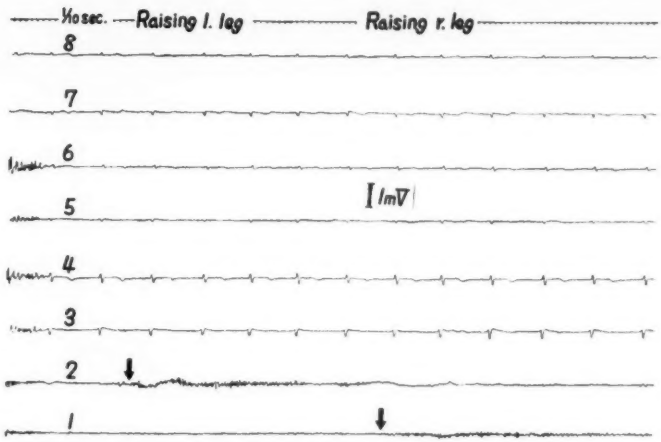

Fig. 3. Muscle-action potentials to show the pattern of activity in the supine position for raising the left or right leg. The action potentials are recorded from the ipsilateral internal obliques.

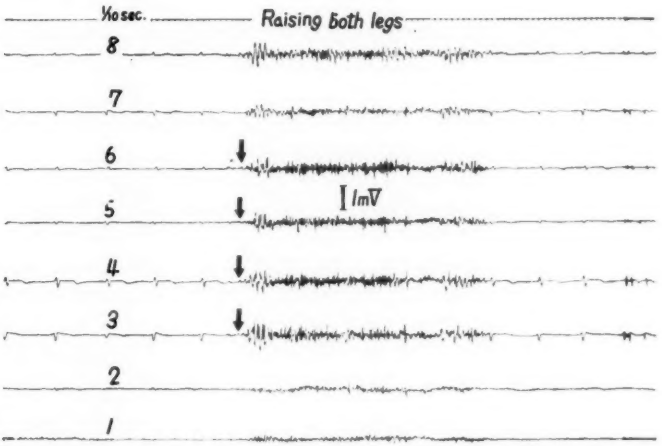

Fig. 4. Muscle-action potentials to show the pattern of activity for raising both legs from the supine position. All abdominal muscles are in action. Especially remarkable in the recti and external obliques.

oblique activity is stronger than that of the external obliques. But the discharges from the recti are slight (Fig. 5). Even when a maximal effort is made, the rectus activity is much less than that recorded from the obliques, and less also than the activity recorded from the same rectus when the head is raised. So the oblique muscles are essentially different

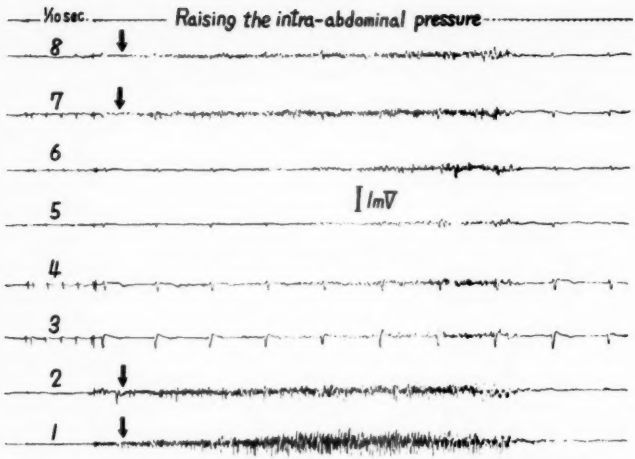

Fig. 5. Muscle-action potentials to show the pattern of activity in the supine position for raising the intra-abdominal pressure. The external and internal obliques are mainly involved.

from those of the rectus abdominis in this function. It seems, that the internal and external obliques play an active role in protecting the inguinal region from the downward displacement of the intra-abdominal viscera caused by increasing the intra-abdominal pressure. That is to say, there is a close relationship between the abdominal pressure and the oblique muscles.

When the subjects are at rest in a standing posture, the internal oblique fibres are in a state of slight contraction. The contraction recorded from the external obliques and recti may be modified by movement backwards and forewards or from side to side, but the contraction of the internal oblique fibres is continuous. This resting discharge from the internal oblique was clearly recorded by the application of high amplification (Fig. 6). The amplitude of this discharge was 50–100 μV. This degree of resting tone normally exceeds that of the other muscles of the abdominal wall. When the abdominal pressure is increased, the tone in these fibres markedly increases. As to this phenomenon, Floyd and Silver presumed that these fibres were continually on guard, and protected the inguinal region from rupture. In any case, it appears that the continuous contraction of the internal oblique fibres is being done as a result of the stretch reflex which is caused by the weight of the intra-abdominal viscera.

Discussion

In the electromyographic experiments mentioned above, a brief

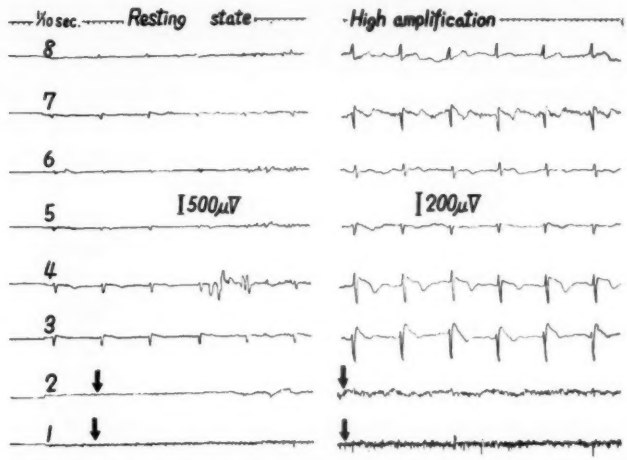

Fig. 6. Subject standing upright. Note a slight contraction of the internal obliques in the resting state. The right half shows the same record taken at high amplification.

general survey was made with regard to the patterns of activity of the abdominal wall muscles in normal adults. As yet, there are no good methods available for recording several physiological phenomena simultaneously. The method devised by Floyd and Silver precludes the possibility of simultaneous recording of the activity of the abdominal wall muscles. The ink-writer oscillograph has an advantage in that the eye can be used to judge the pattern of activity for any movements. As stated previously, it is convenient to use the surface electrodes which pick up the algebraic sum of the action potentials of all active motor units in the muscle for the purpose of this study. But the parts of the muscle more remote from the electrodes have a smaller effect than the adjacent parts.

The electrical record shows that the rectus is primarily involved when the head is raised. The fundamental pattern for increasing the intra-abdominal pressure, however, is the antithesis of the head lifting pattern and mainly involves oblique muscles, especially the internal oblique other than the rectus. The internal oblique muscles play a prominent role in this movement. Even when a maximal effort is made in supine position, the rectus activity is only slightly increased. It seems that the rectus activity becomes evident only at the time when a flexion of the trunk causes the movement that raises the intra-abdominal pressure.

The continuous activity of the internal oblique in erect posture is perhaps an expression of the effort of the internal oblique, by which the intra-abdominal viscera are supported against gravity. It seems there-

fore that the internal obliques play an important part as the anti-gravity muscle in the abdominal wall. On the other hand the rectus muscles are less helpful for supporting viscera as the anti-gravity muscle. This is an interesting matter.

The above-mentioned results may be able to confirm and amplify the classical account of the functional anatomy of the abdominal wall muscles.

Summary

An electromyographic study on the abdominal wall muscles in normal adults was performed by the use of surface electrodes after Floyd and Silver with eight-channel electroencephalograph. The subjects employed were 15 healthy adults, 10 males and five females, ranging in age from 17 to 40 years.

Results obtained are as follows:

1. Head raising gives rise to the dominant activity of the rectus in supine posture. When the intra-abdominal pressure is raised in supine posture the action of the oblique muscles, especially of the internal, is clearly observed. The rectus plays a passive role and is only involved when the trunk is flexed at the same time. It is, therefore, evident that there is a close relationship between the intra-abdominal pressure and the internal obliques.

2. When the subjects are at rest in a standing posture, the internal oblique muscles demonstrate a state of slight activity.

The amplitude of this discharge is 50–100 μV. This phenomenon indicates that this continuous activity of the internal obliques is being done as a result of the stretch reflex which the weight of the intra-abdominal viscera provokes on the internal obliques. It seems, therefore, that the internal obliques play an important role as an anti-gravity muscle supporting the intra-abdominal viscera.

References

1) Campbell, J. Physiol., 1952, **117**, 222.
2) Campbell & Green, *ibid.*, 1953, **120**, 409.
3) Campbell & Green, *ibid.*, 1953, **122**, 282.
4) Campbell, *ibid.*, 1957, **136**, 556.
5) Takano, Jap. J. Physiol., 1956, **6**, 22.
6) Nakano, Nanjo, Komiyama & Chiba, Tohoku Igaku Zassi (Jap.), 1952, **47**, 283.
7) Nanjo, Kawamura, Komiyama, Yamazaki, Watanabe & Hashimoto, *ibid.*, 1955, **51**, 78.
8) Komiyama, Nanjo, Yamazaki & Watanabe, *ibid.*, 1955, **52**, 461.
9) Kawakami, Jap. J. Physiol., 1954, **4**, 274.
10) Floyd & Silver, J. Anat., 1950, **84**, 132.

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Electromyographic Studies of the Abdominal Wall Muscles in Visceroptosis

II. Peculiarity of the Abdominal Wall Muscles in Visceroptotic Subjects

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In the preceding paper[1], an electromyographic study of the patterns of activity of the normal human abdominal wall muscles was described. Since early times, it has been emphasized[2)-4)] that the laxity of the abdominal wall had an important significance as an etiologic factor of visceroptosis. Therefore, an electromyographic investigation of the abdominal wall muscles was made of patients with visceroptosis. The results obtained are presented in this paper.

Experimental

Method

Thirty patients of visceroptosis, 11 males and 19 females, aged 26 to 45, who had been clinically diagnosed in our clinic were examined. The apparatus, technique and procedure employed were almost the same as that described in the previous paper.

Results

In supine posture, the patterns of activity of the abdominal wall muscles of visceroptosis for the various movements do not differ essentially from those of the healthy subjects. But the contraction of the internal obliques in visceroptosis is more conspicuous than normal when the raising of the intra-abdominal pressure is caused (Fig. 1). From this phenomenon, it appears that the internal obliques of the visceroptotic subjects must be more actively involved than in normal over in supporting the viscera against gravity, because the downward displacement of the viscera may take place to a high degree in them when the intra-abdominal pressure is increased. The continuous resting discharge recorded from the internal obliques in standing posture may be an interesting question as an expression

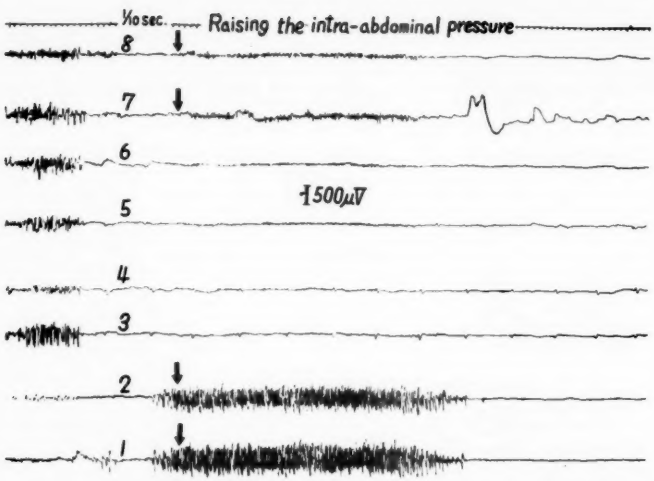


Fig. 1. Muscle-action potentials to show the pattern of activity of a visceroptotic subject in the supine position or raising the intra-abdominal pressure. Note the chief involvement of the internal obliques and the minimal involvement of the recti.

of its function which supports the viscera against gravity. Because visceroptosis is a disease of the human race, in which the erect posture is the basal responsible factor.

The degree of the contraction of the muscle as a whole can be roughly estimated by the amplitude of the discharge, if surface electrodes are used for recording. The average value of the amplitudes of this resting discharge from the internal obliques was measured in every case, and a comparison was made between normal and visceroptotic subjects. The results are shown in Table I. As the table indicates, the amplitudes of visceroptotic

TABLE I

Comparison of Amplitudes of Resting Discharges Recorded from Internal Obliques in Standing Position between Normal and Visceroptotic Subjects

Amplitude (μV)	below 50	50–100	100–200
Normal Subjects	5	7	3
Visceroptotic Subjects	3	8	19

subjects are chiefly from 100 to 200 μV, while those of normal subjects are mostly less than 100 μV. Fig. 2 shows an example of continuous resting

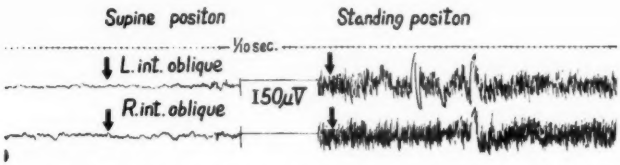


Fig. 2. Visceroptotic subject in the resting state. The resting discharge from the internal obliques in standing position is very conspicuous.

discharge from the internal obliques of visceroptotic subjects when they are standing upright.

At the same time, a fluoroscopic examination of the alimentary tract was performed on these subjects. Fig. 3 is a lateral view showing that most loops of the small intestines have fallen into the pelvic cavity and the

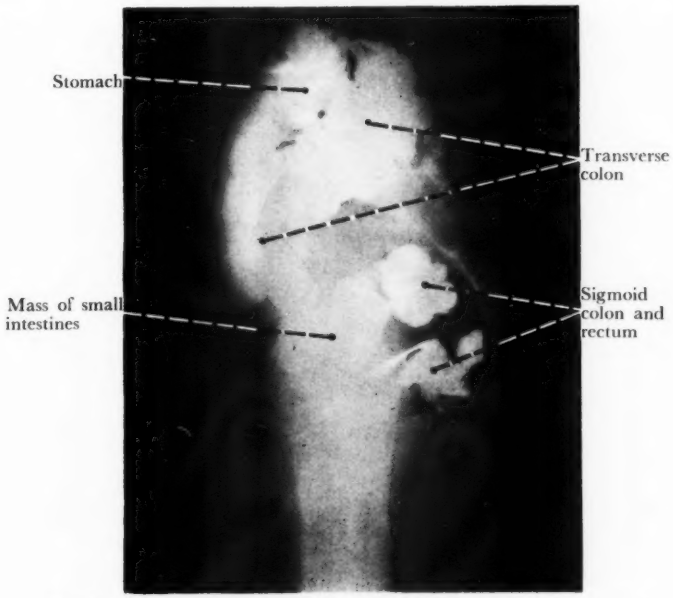


Fig. 3. Lateral radiograph of a visceroptotic subject with opaque meal. This demonstrates that the direct cause of the discharge from the internal obliques is the leaning of the stomach and colon against the lower abdominal wall from inside the abdominal cavity.

markedly ptotic transverse colon and the stomach are located under the abdominal wall. This explains, therefore, that a direct cause of the resting discharge from the internal obliques seems to be caused by the stretch

reflex of these muscles as a result of carrying the direct weight of the stomach and the transverse colon. But the indirect effect of the mass of the small intestines can not be overlooked. Fig. 4 shows the pre- and post-operative states of the resting discharge in erect posture from the internal obliques of a patient of gastrocoloptosis who has had a gastrectomy and colectomy. The discharge which was remarkable before the operation is markedly reduced or diminished after the operation. This clearly demonstrates that the internal obliques no longer support the direct weight of the stomach and the transverse colon.

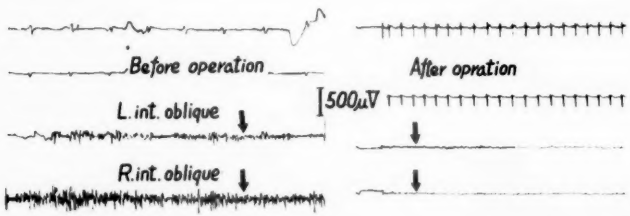


Fig. 4. This shows the pre-and post-operative states of the resting discharge erect posture from the internal obliques of a patient of gastrocoloptosis who has had gastrectomy and colectomy.

When the subjects are changed to the knee elbow posture from the erect posture, the resting discharge becomes little as that in the supine posture (Fig. 5). This fact, perhaps, indicates that the load of the stomach

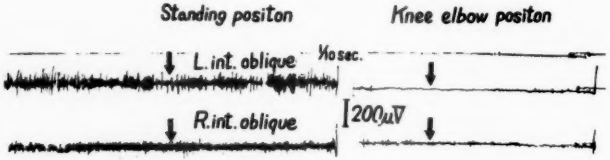


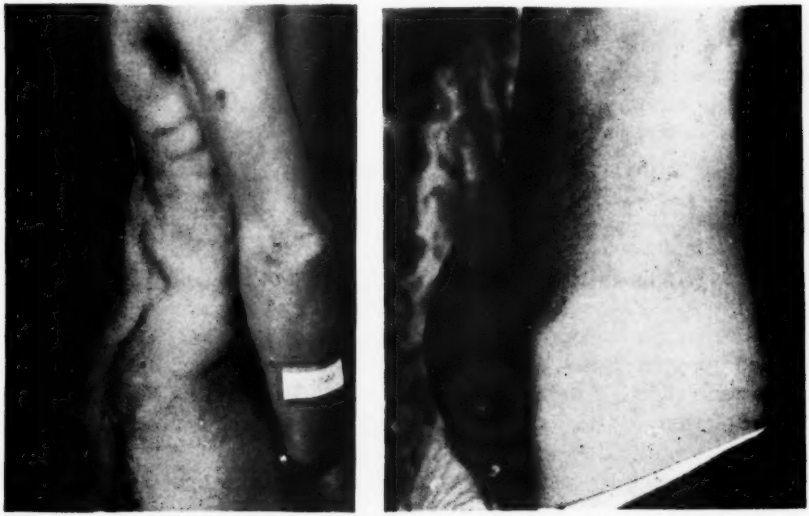
Fig. 5. The resting discharge from the internal obliques becomes little as that in supine position by the taking knee elbow position.

and the bowels on the internal obliques are widely dispersed over the the anterior abdominal wall so as not to cause a rise in the resting discharge.

Discussion

Any or all of the organs situated below the diaphragm may be found below their normal level when the body is in the erect posture, the assumption of which by the human race is the basal responsible factor. The

predisposing causes are congenital laxity of the visceral supports, weakness of the abdominal wall, downward pressure upon the subdiaphragmatic organs from habitual faulty posture and clothing which constricts or drags upon the waist. When the degree of prolapse is appreciable, the term visceroptosis has been applied to the condition, and it is now recognized that serious disturbances of normal body functions may result from it. A useful division into congenital and acquired types may be made. Stiller[5),6)] emphasized the significance of the congenital type in visceroptosis advocating the term Habitus enteroptoticus s. paralyticus or Asthenia universalis. This type is met with among the physically degenerate, and results from defective development. The sufferers from it have never at any time been normal and in this respect differ from those with the acquired type, the latter at some period of their life enjoyed ordinary health. Acquired visceroptosis is very common, especially among women. Fig. 6



A 40-year-old man A 34-year-old woman

Fig. 6. Epigastric region is depressed. The lower abdomen shows undue prominence.

demonstrates two typical instances of these cases. This form of visceroptosis is most marked in women who have borne many children and whose abdominal walls are lax and pendulous. From this point of view Landau[1)] attached great importance to the attitude of the anterior abdominal wall muscles. According to Rovsing[7)], this type was called "Landausche Form der Ptose" or "maternelle Ptose."

As a matter of fact, the abdominal cavity is a large nonskeletal space between the caudal margin of the thorax and cranial margin of the pelvis, as described in anatomical texts " Lacuna sceleti sterno-pubica Rauber."[8] The role of the abdominal muscles is very important, not only for the support and protection of the intra-abdominal viscera, but also for the control of the intestinal movement. Visceroptosis is a term applied to prolapse or falling of the various viscera. Therefore, the weakness and relaxing of the abdominal wall muscles, supporters and protectors of the viscera, have an important significance as a cause of visceroptosis. In the experiments mentioned above, it is conceivable that the continuous resting discharge from the internal obliques in erect posture is an expression of some degree of prolapse or falling of the bowels into the lower abdominal wall. The lateral radiograph (Fig. 3) endorses this fact. It is, therefore, reasonable to assume that the degree of visceral prolapse can be estimated by the amplitude of the internal oblique discharge using surface electrodes. That is to say, the degree of the load on the internal obliques can be semiqnantitatively estimated by this method. In visceroptosis, according to the results obtained, the resting discharge is characterized by waves having an amplitude of 100–200 μV, while the normal is below 100 μV. This apparently reveals that the activity of the internal obliques which endeavours to support the bowels is more conspicuous in visceroptosis than in normal, because the downward displacement of the bowels is greater in the former than in the latter. As a cause of visceroptosis, Glénard[9] (1899) emphasized the significance of movability of the Flexura colicohepatica, from which the downward prolapse of the colon, stomach and the other viscera resulted. However, it may also be necessary to have in mind that the mass of the small intestines which fell into the pelvic cavity pulled down the Radix mesenterii causing the ptosis of the stomach, duodenum and colon. From this fact, it is easy to understand that the direct cause of the discharge is the leaning of the colon and stomach against the internal obliques from inside the abdominal cavity as shown in Figs. 3 and 4.

The resting discharge from the internal obliques in erect posture is almost diminished to the same degree as that in supine posture, by taking the knee elbow posture. The knee elbow posture is essentially the same as that of the four-footed animals among which there exist no visceroptosis. Furthermore, the clinical symptoms of the arterio-mesenteric duodenal stenosis[10]–[15] which is recognized as a phenomenon of visceroptosis are markedly improved by taking the knee elbow posture. Thus, as to the experiment described above, it can be suggested that the disappearance of the discharge from the internal obliques in knee elbow posture is closely related to these facts.

SUMMARY

An electromyographic investigation on the abdominal wall muscles in visceroptotic subjects was performed using an eight channel electroencephalograph with surface electrodes in 30 cases of visceroptosis. The results obtained are as follows:

1. In visceroptosis, the resting discharge from the internal obliques in erect posture is more than normal; the waves of discharge have an amplitude of 100–200 μV in these cases, while below 100 μV in normal.

2. This is apparently due to the fact that in visceroptosis the internal oblique which support the intra-abdominal viscera, especially stomach and colon, may have to work harder than in normal.

3. It may be, therefore, impossible to discuss the pathologic physiology of visceroptosis without asking about the anti-gravity function of the internal oblique muscles.

References

1) Ono, Tohoku J. Exp. Med., this volume, p. 347.
2) Landau, Die Wanderleber und der Hängebauch der Frauen, August Hirschwald, Berlin, 1885, 64.
3) Quincke, Therap. Gegenw., 1905, 3.
4) Billington, Movable kidney, 2.ed., Cassel & Company, London, 1929, 9.
5) Stiller, Die asthenische Konstitutionskrankheit, Ferdinand Enke, Stuttgart, 1907, 11.
6) Stiller, Grundzüge der Asthenie, Ferdinand Enke, Stuttgart, 1906, 7.
7) Roving, Cited from Bergmann's Handbuch der inneren Medizin, Vol. 3, 2.ed., Julius Springer, Berlin, 1928, 198.
8) Eisler, Die Muskeln des Stammes, Gustav Fischer, Jena, 1912, 56.
9) Glénard, Cited from Boas' Diagnostik u. Therapie der Magenkrankheiten, 7.ed., Georg Thieme, Leipzig, 1920, 570.
10) Schnitzler, Wien. Klin. Rundschau, 1895, 579.
11) Albrecht, Virchows Arch., 1899, **156**, 285.
12) Haberer, Erg. Chir., 1913, **5**, 467.
13) Haberer, Arch. klin. Chir., 1924, **132**, 191.
14) Koennecke & Meyer, Dtsch. Zschr. Chir., 1922, **175**, 179.
15) Halpert, Virchows Arch., 1923, **244**, 439.

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Electromyographic Studies of the Abdominal Wall Muscles in Visceroptosis

III. Therapeutic Significance of the Abdominal Bandage for Visceroptosis

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(Received for publication, July 3, 1958)

In preceding papers[1)2)], it has been stated that the activity of the internal obliques which support the intra-abdominal viscera when the body is erect has an important significance for the pathologic physiology of visceroptosis. Concerning the treatment for visceroptosis, there may be a need to reinforce the antigravity function of the internal obliques. There are several methods employed for the treatment of visceroptosis, such as abdominal bandages, bodily exercise to strengthen the abdominal muscles, and surgical methods. The most widely used treatment has been the abdominal bandage and it is generally recognized that the symptoms of visceroptosis have been improved to some degree by the application of a suitable bandage. Why does the bandage give a good result in cases of visceroptosis? It is commonly thought[3)] that this therapeutic effect is due to the elevation of the ptotic stomach and bowels. However, as a matter of fact, the fluoroscopic examination revealed that the stomach was not much elevated even when the subject wearing the abdominal bandage felt comfortable in his abdomen. And as for his bowels which had fallen into the pelvic cavity, they were not at all elevated and rather their position was further lowered.

Then with the object of inquiring into the reason why the abdominal bandage is effective for the treatment of visceroptosis, some electromyographic studies of the abdominal wall muscles, especially of the function of the internal obliques were performed. The results obtained will be presented.

Experimental

Method

For this purpose, 15 cases of visceroptosis and ten healthy subjects

were chosen. An eight channel amplifier with ink-writer oscillographs was used and action potentials from the abdominal wall muscles were recorded using surface electrodes as described in the previous papers. An abdominal bandage made of cotton, 15×250 cm. in size, including a cuff connecting to the sphygmomanometer, was used. In the first instance, the subjects wore the bandage so that the cuff might sit exactly on their hypogastrium and in the next instance on their epigastrium after placing the electrodes over the anterior abdominal wall as described previously. Then the reaction to various degrees of pressure of the abdominal wall muscles in erect posture was electromyographically examined.

Results

a) Wearing the bandage on the hypogastric region

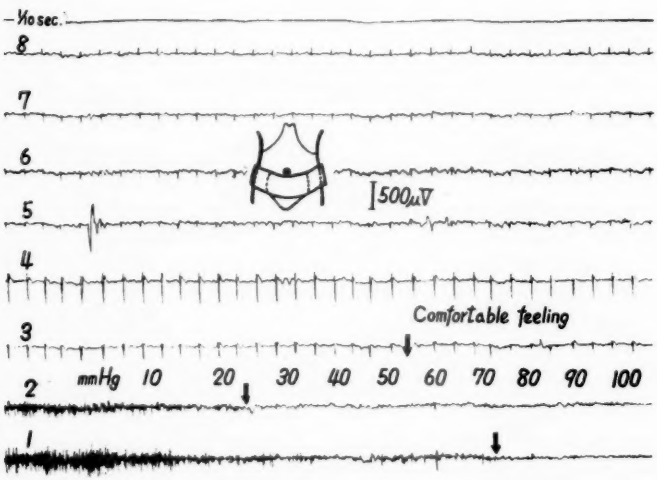

Fig. 1. Wearing thr bandage on the hypogastric region. Subject erect and quiet. The resting discharge from the internal obliques is gradually diminished with the increase of pressure of the cuff in the bandage. The subject felt comfortable in his abdomen at the level of 50–60 mm. Hg.

In the instance of no pressure increase, the resting discharge from the internal obliques is seen in erect posture. This may be a result of the burden of the intra-abdominal viscera on the internal obliques as described in preceding papers. But this discharge is gradually diminished with the increase of pressure on the lower abdominal wall. When the increase of the pressure averages more than 20 mm.Hg., the activity of the internal obliques drops too low to be recorded (Fig. 1). When the pressure is

increased, the subject feels more comfortable in his abdomen. At this time, the manometric range is between 20 and 70 mm. Hg. This distribution of the subjects according to the point at which comfort is felt is shown in the accompanying table. When the increase in pressure is

TABLE I

Distribution of Subjects According to Point at which Comfort is felt by Raising Pressure of Cuff in Bandage

Pressure of the cuff (mm. Hg.)	Normal subjects	Visceroptotic subjects
20—30	4	3
31—40	3	3
41—50	1	7
51—60	1	2
61—70	1	0

continued, the subject presently begins to complain of pain in his abdomen again. The manometric reading of this time is generally more than 70 mm. Hg., and there is still no recorded activity of the internal obliques.

At the same time, a fluoroscopic examination of the stomach and bowels was performed. Fig. 2 shows the dorso-ventral radiograph of the abdomen in erect posture without wearing the bandage. The stomach is slightly ptotic and the transverse colon has fallen into the pelvic cavity. When the subject takes a supine posture, his stomach is markedly higher and his transverse colon is also slightly higher (Fig. 3). However, when the same subject wears the bandage on his lower abdomen in erect posture and feels comfortable in his abdomen or even when he suffers from pain by excessive increase of the pressure, the stomach only is slightly elevated, and the downward displacement on the colon is rather promoted (Figs. 4 and 5).

When the pressure from the bandage is reduced the resting discharge appears again as before. At the same time, the comfortable feeling disappears and the patient becomes aware of a heavy feeling or sense of fullness in his abdomen of which he complained at first. This sensation is one of the chief complaints of the visceroptotic subjects when in an erect posture (Fig. 6). No variations in the activities of the other muscles are seen.

b) Wearing the bandage on the epigastric region

This experiment was designed to discover the effect upon the intra-abdominal viscera from the use of unsuitable corsets or belts. When the pressure of the cuff is gradually increased, the internal oblique activity becomes stronger in contrast to the former experiment. The maximal

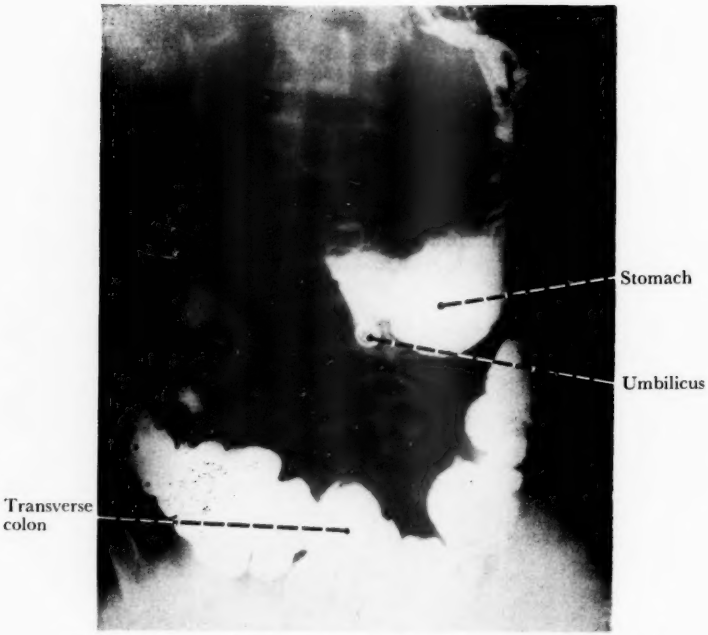

Fig. 2. Radiograph of a patient in erect posture without wearing the bandage. The stomach is slightly protic and the transverse colon is fallen into the pelvic cavity.

amplitude of the discharge is recorded in the range of 400 to 500 μV at the time when the pressure is heightened to about 70 mm. Hg., and the pain becomes intolerable in the subject's abdomen. When the pressure is decreased, the discharge from the internal obliques again weakens and returns to its previous level. The discharges from the other muscles also become intense as the pressure is increased. Fig. 7 shows this phenomenon.

Discussion

Many kinds of abdominal bandages[4)-7)] have been widely employed since early times as a most useful method for the treatment of visceroptosis. It is commonly thought that the bandages give firm support to the lower abdomen and generally " lift up " the viscera. The experiment mentioned above, however, clarified the fact that the " lift up " theory[3)] might not be strictly true. By the use of the bandage on the lower abdomen, the stomach will be slightly elevated, but contrary to expectations the bowels may be further depressed into the pelvic cavity. However, the subject

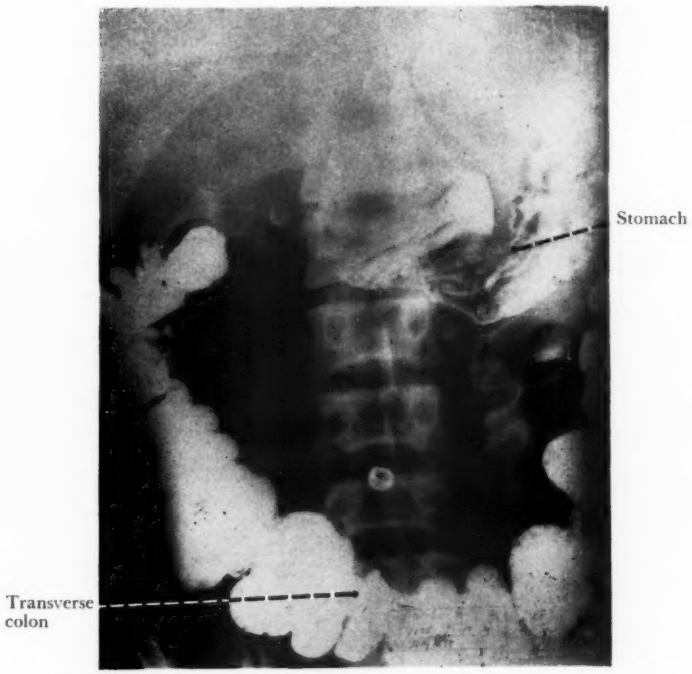

Fig. 3. The same subject in supine posture. The stomach is markedly higher and transverse colon is also slightly higher.

feels comfortable in his abdomen with this bandage, which is almost the same feeling as that obtained by anyone who applies moderate pressure to his lower abdomen. Moreover the above described experiment revealed that the internal oblique discharge ceases with the increase of the pressure. The significance of this phenomenon may be that the anti-gravity function of the internal obliques is reinforced by the bandage, namely, the bandage takes the internal obliques place as a supporter of the viscera. Therefore, it may be said that the abdominal bandage is of great use for the reinforcement of the anti-gravity function of the internal obliques rather than for the elevation of the viscera. There are several reports[8)-13)] in which the effect of the bandage on visceroptosis was studied from the point of view of blood circulation. So pressure moderately applied to the lower abdominal wall may reinforce the internal obliques resulting in a good neurogenic or circulatory effect on the physiologic condition of the intra-abdominal viscera. Anyway, the reinforcement of the internal oblique function as a supporter of the viscera is most important for the correct

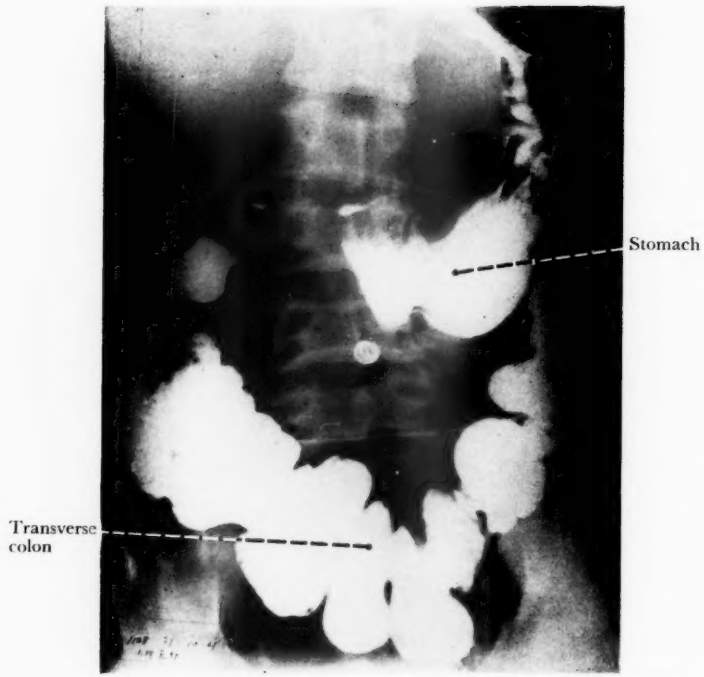

Fig. 4. The same subject wearing the bandage on the hypogastric region in erect posture. He felt comfortable in his abdomen by raising the pressure of the cuff to 50 mm. Hg. The stomach only is slightly elevated, but the downward displacement of the colon is rather promoted.

treatment of visceroptosis. Thus, when visceroptosis is treated by the bandage method, it is necessary to apply the pressure at the proper place and use moderate pressure so that both internal obliques may be correctly reinforced.

Pressure on the upper abdominal wall causes an abnormal increase of the resting discharge from the internal obliques as shown in the experiment. This fact indicates perhaps that the intra-abdominal contents are displaced downward by the bandage applied on the upper abdomen, and consequently the internal oblique must exert more effort to support the viscera. That is to say, visceroptosis will be intensified by the applying of pressure to the upper abdomen. This experiment seems to give endorsement to the " Corset theory "[14)-17)] which has been emphasized as a cause of visceroptosis since early times. From this point of view, it may also be said that visceroptosis is aggravated by the use of an unsuitable girdle or belt or the upward slipping of the bandage from the lower abdominal wall.

Fig. 5. He suffers from pain by excessive increase of the pressure (about 160 mm. Hg). The state of the stomach and the bowels are almost the same as Fig. 4.

Attention should be given to these facts when visceroptosis is to be treated with a bandage.

Summary

For the purpose of inquiring into the therapeutic significance of the abdominal bandage for visceroptosis, an electromyographic investigation on the abdominal wall muscles was performed using surface electrodes and an eight channel electroencephalograph. The subjects employed were fifteen cases of visceroptosis and ten healthy adults.

Results obtained are as follows:

1. The therapeutic significance of the abdominal bandage for visceroptosis exists in the reinforcement of the anti-gravity function of the internal obliques rather than lifting up the intra-abdominal viscera.

2. When visceroptosis is to be treated with a bandage, it will be necessary to determine the suitable part of the abdominal wall on which to apply moderate pressure so that the internal obliques will be correctly

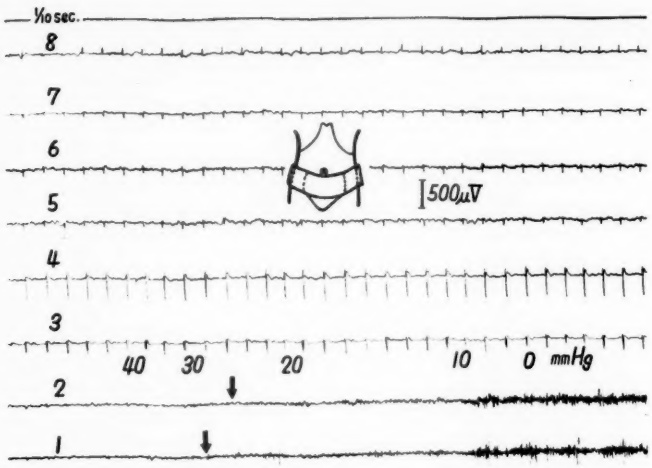

Fig. 6. Wearing the bandage on the hypogastric region. Subject erect and quiet. The resting discharge from the internal obliques appears again as before with the reducing pressure of the cuff.

reinforced.

3. The symptoms of ptosis are intensified by the applying of pressure on the upper abdominal wall. Namely, the " Corset theory " which had been considered as a cause of visceroptosis since early times was given its endorsement by this experiment.

4. Therefore, the use of unsuitable corset and belt, or upward slipping of the bandage from the lower position are opposed to the purpose of treatment for visceroptosis.

References

1) Ono, Tohoku J. Exp. Med., this volume, p. 347.
2) Ono, *ibid.*, this volume, p. 355.
3) Borgbjärg & Fischer, Arch. Verdauungskrkh., 1912, **18**, 441.
4) Clemm, Therap. Gegenw., 1904, 187.
5) Glemm, *ibid.*, 1909, 213.
6) Gallant, J.A.M.A., 1908, **51**, 1568.
7) Burckhardt, Erg. Chir., 1912, **4**, 285.
8) Hill, J. Physiol., 1895, **18**, 15.
9) Hering, Münch. med. Wschr., 1927, **74**, 1611.
10) Laurell, Fortschr. Röntgenstr., 1936, **53**, 501.
11) Tomita, Nihon Shokakibyo Gakkai Zassi (Jap.), 1941, **40**, 181.
12) Takahashi, Kumamoto Igk. Z. (Jap.), 1943, **19**, 1507.
13) Takahashi, *ibid.*, 1943, **19**, 1953.
14) Cruveilhier, Cited from Bergmann's Handbuch der inneren Medizin, Vol.3, 2.ed., J.

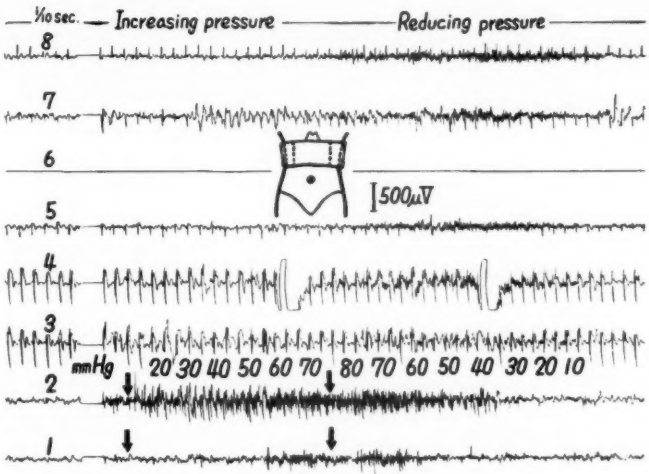

Fig. 7. Wearing the bandage on the epigastric region. Subject erect and quiet. The internal oblique activity becomes stronger by the increase of pressure of the cuff, but again weakens and returns to its previous level with the decrease of pressure.

Springer, Berlin, 1926, 197.

15) Müller-Warneck, Berl. Klin. Wschr., 1877, 429.

16) Koranyi, *ibid.*, 1890, 702.

17) Fleiner, Münch. med. Wschr., 1895, **42**, 973.

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Histopathological Findings on a Function of Bronchiolus Respiratorius

II. Mechanism of Dust-macule Formation*

By

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Simon[1] has already pointed out that silicotic nodules and dust maculae come into formation preferentially in the area where the end of the bronchiolus respiratorius branches out into ductus alveolaris, upon study by reconstruction, but by what process such nodules are formed in this area is not yet clarified. In the past, it was generally assumed that dust deposited on the alveolar septa is carried out through the tissue interstices or by lymph flow and accumulated in the lymphoid tissue existing in the peripheral area of the bronchioli respiratorii (Miller[2]). But this opinion, though widely accepted, is nothing but a hypothesis.

In a previous study[3], We have found the dust inhaled by the lung accumulated rather less in the alveoli belonging to the sacculi alveolares than in the alveoli more proximal to them, namely, in the alveoli projecting from the walls of the alveolar ducts and of the respiratory bronchioles, and have reported that this finding may give a clue to the elucidation of the mechanism of nodule formation (Fig. 1) in pneumoconiosis. With the aim of corroborating this opinion, we made further examinations of pneumoconiosis cases, and succeeded in clarifying the process of such nodule formation in the peripheral range of the bronchioli respiratorii.

Experimental

Materials and Method

We selected comparatively normal lungs of adults showing no marked pathological change out of autopsy specimens in general, and then mild cases out of the pneumoconiosis patients from Jōban, Osaruzawa and Sado

* Read at the 47th General Meeting of the Japanese Pathological Society held at Osaka on 3 April 1958.

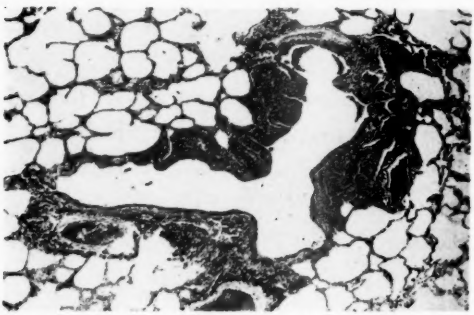

Fig. 1. The initial stage of silicosis. Nodules are formed in the wall of the respiratory bronchiolus and the alveolar duct.

Mines, for grasping the picture of the initial stage of nodule formation.

The specimens were paraffin embedded, cut into serial sections and stained with H-E, by Weigert's elastica staining, Goldner's staining and Gomori's silver impregnation methods, for microscopic examination.

Results

As stated above, the degree of intra-alveolar accumulation of dust is not uniform everywhere, but more in the alveoli projecting from the walls of the alveolar ducts and of the bronchioli respiratorii (alv. br. for short) rather than in the alveoli belonging to the sacculi alveolares. The dust filling some alveoli seemingly unconnected with the alv. br. is found to originate in dust accumulated in the alv. br., when we look through the serial sections (Fig. 2, 3). Whereas a rather large quantity of dust is

Fig. 2

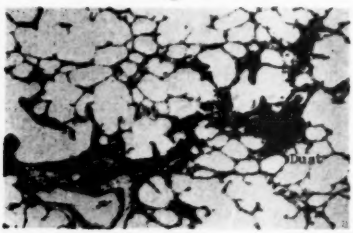

Fig. 3

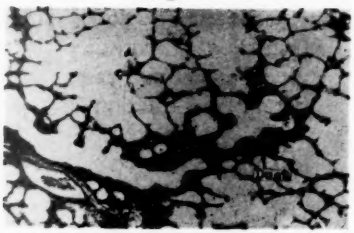

Fig. 2, 3. Dust accumulation in normal lung. Seemingly, the dusts accumulate in the alveoli belonging to the sacculus alveolaris but it is found that the dusts accumulate in the alveoli projecting from the wall of the bronchiolus respiratorius and the ductus alveolaris (alv. br. for short), when we look through the serial sections.

found in the lumina of the alv. br. of normal lungs too, very little dust is

found in the alveoli of the sacculi alveolares in such cases, showing a distinct difference. This finding is probably attributable to the fact that while the dust inhaled into the saccular alveoli is readily expelled by respiratory movement of the lung, the alv. br. are attached to the respiratory tract in a vertical position to it, so that the dust in it is apt to remain accumulating in them.

In coincidence with the picture of dust accumulation in the alveolar lumina, dust was found deposited on the septa of alv. br. too. This picture tells that the dust accumulated in the alv. br. has made way into the interalveolar stroma in situ.

Such dust particles were mostly found taken into the cell bodies, whether in the alveoli or the stroma between them, and the septa of the alv. br. with deposited dust showed proliferation of argyrophil fibres in a mild degree and were found thickened (Fig. 4). On the other hand, where

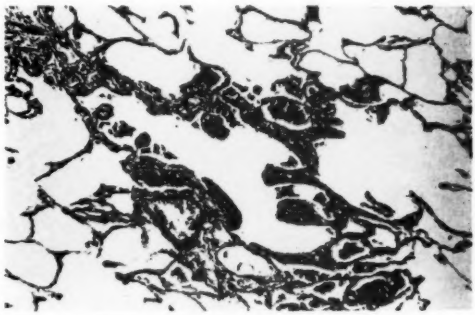

Fig. 4. Normal lung. Dusts accumulate in the lumina and septa of the alv. br. and proliferation of argyrophil fibres in a mild degree is found with the thickening of the alveolar septa.

dust remained accumulated within the alveoles only, no fibres formation was apparent.

Upon following up this process in cases of pneumoconiosis of the initial stage, such a finding was more apparent and in particular, the lumina of the alv. br. were found filled up with a large quantity of dust. Much dust was found deposited in the septa of the alv. br. and argyrophil fibres were found proliferated and entwining the deposits (Fig. 5). Consequently, the alveolar septa were thickened and the alveolar lumina pressed down in width. In the areas where this process was further advanced, the septa of the alv. br. had incrassated further with the increase of dust accumulation, and between them much narrowed-down lumina were visible. In the Figs. 5 through 8, this process is illustrated, and we can see the alveolar lumina filled with dust remaining between the thickened septa.

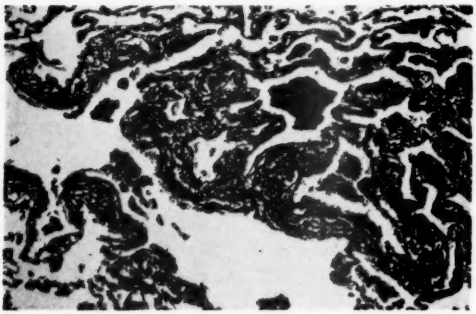

Fig. 5. Pneumoconiosis case. Much dust is found deposited in the septa of the alv. br. and argyrophil fibres are found proliferated and entwining the deposits.

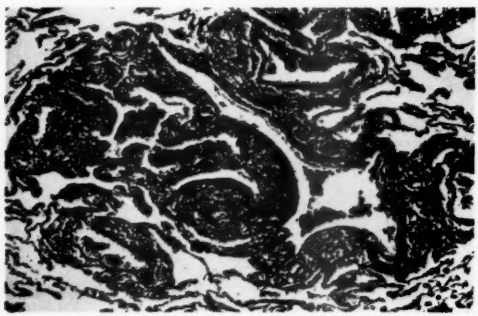

Fig. 6. Pneumoconiosis case. The thickening of the septa is further advanced.

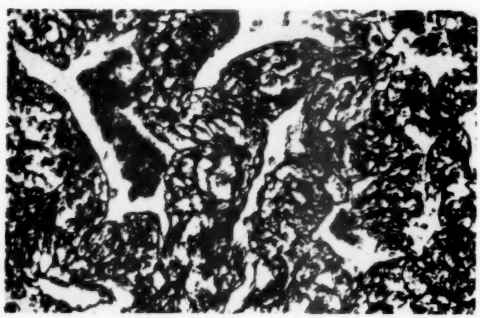

Fig. 7. Pneumoconiosis case. The septa of the alv. br. are thickened further and between them much narrowed-down lumina are visible.

Finally, the swollen septa come to touch each other and the lumina are narrowed down to hair-like slits between them or closed down alto-

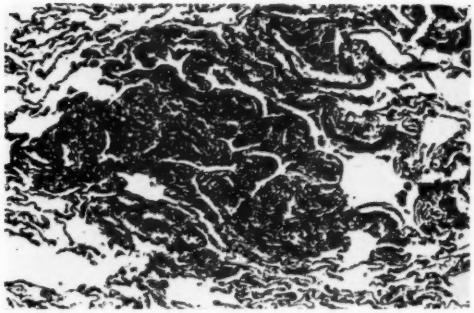

Fig. 8. Pneumoconiosis case. The swollen septa come to touch each other and the lumina are narrowed-down to hair-like slits between them or closed down altogether, wherein the thickened septa form a nodular mass in the whole.

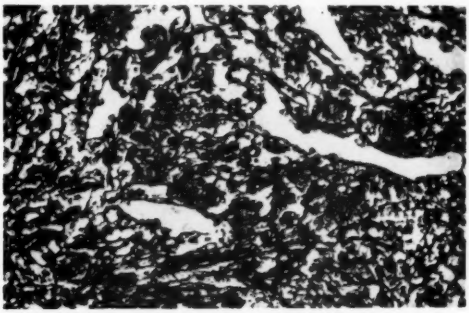

Fig. 9. Pneumoconiosis case. Small gaps in the nodulus show the rests of the alveolar lumina.

gether, as shown in Fig. 8, 9, wherein the thickened septa form a nodular mass in the whole, with small gaps left here and there. In this way, pneumoconiotic nodules are formed in the region of the bronchioli respiratorii. Fig. 10 shows a completely developed pneumoconiotic nodule under common staining. The relics of the alveolar lumina are found as slits, endorsing the above-described process.

Conclusion

In pneumoconiosis, dust maculae and silicotic nodules primarily form in the lymph nodes of the hilus and in the peripheral part of the lungs. The present authors made microscopic examination of specimens from early pneumoconiosis cases and succeeded in clarifying the process of nodule formation in the peripheral part of the lungs, in summary as follows: The dusts inhaled into the lung mostly accumulate in the alveoli projecting from the wall of the alveolar ducts and of the respiratory bronchioles and

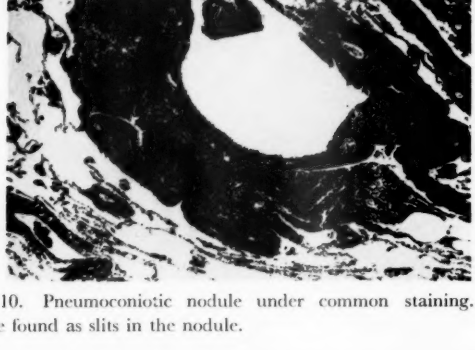

Fig. 10. Pneumoconiotic nodule under common staining. Alveolar lumina are found as slits in the nodule.

invade the alveolar stroma in situ. A part of them is carried off in the lymph flow and induces pneumoconiotic changes in the lymph nodes of the hilus, but the remainder frankly accumulate in the stroma to induce marked thickening of the alveolar septa in this region, which finally fuse together, blotting out the alveolar lumina and completing the nodule formation.

References

1) Simson, F. W., J. Path. & Bact., 1935, **40**, 37.
2) Miller, W. S., The lung, 2. ed., Springfield, 1950.
3) Takizawa, T. & Haseyama, H., Tohoku J. Exp. Med., 1957, **66**, 24.

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On the Spinal Root Pathway of the Adrenaline-Secretory Fibers to the Adrenal Gland in Toads

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(Received for publication, July 10, 1958)

The pathway of the adrenaline-secretory fibers to the adrenal gland in mammals has been studied by many investigators. We are, however, fully unaware of that in cold blooded animals such as frogs and toads.

In my previous study[1] it was observed that the marked depletion of the adrenaline content of the adrenal gland in toads was producible by insulin hypoglycemia and this depletion was abolished by the previous section of the splanchnic nerves in the corresponding side. These experimental results indicate that the adrenaline-secretory fibers to the adrenal run in the splanchnic nerves.

The present investigation was undertaken to determine the spinal roots through which the adrenaline-secretory fibers course, by measuring the effect of the ventral or dorsal spinal roots sections at various levels of spinal segments on the decrease in the adrenaline content of the adrenal gland causable by insulin hypoglycemia.

Experimental

Methods

Male and female toads were used. All the experimental procedures were almost the same as those of my previous investigation.[1] The spinal roots were laid bare by opening the dura and were sectioned on the left side. Insulin was injected in a dose of 15 units per kg. of body weight. This dose of insulin was found in my previous study to be sufficient for depleting the adrenaline content of the adrenal gland with intact innervation in toads. Since it was proved that the adrenaline content of the splanchnicotomized gland was not influenced by insulin hypoglycemia, the splanchnic nerves were cut on the right side and the adrenaline content of the right gland was taken as the control for comparison. The adrenaline and noradrenaline contents of the gland were estimated by the use of the permanganate method of Suzuki and Ozaki.[2]

Results

(1)

In 7 toads the 3rd–7th dorsal and ventral spinal roots were cut on the left side. The splanchnic nerves were severed on the right side. After 6 days subcutaneous injection of insulin was made and at 10 hours after insulin the adrenaline and noradrenaline contents were estimated.

The adrenaline content of the adrenal gland, computed per kg. of body weight, was 0.182 (0.142–0.215) mg. and 0.188 (0.142–0.218) mg. in the left and the right gland, respectively. That of noradrenaline was estimated as 0.144 (0.120–0.175) mg. and 0.149 (0.108–0.182) mg. in the left and the right gland, respectively. There was no significant difference between the estimates of the left and the right gland ($P>0.25$). Thus the decrease in the adrenaline content producible by insulin was proved to be fully prevented by the section of the 3rd–7th dorsal and ventral spinal roots.

(2)

In 5 toads the left 3rd–7th dorsal spinal roots and the right splanchnic nerves were cut. Six days after the nerve sections the injection of insulin was made.

The adrenaline content per kg. of body weight in the left adrenal gland was measured as 0.107 (0.090–0.130) mg. and that of in the left gland as 0.208 (0.155–0.280) mg. The noradrenaline content per kg. of body weight was 0.114 (0.077–0.162) mg. and 0.118 (0.080–0.160) mg. in the left and the right adrenal gland, respectively. The difference between the estimates of adrenaline content of the left and the right adrenal gland was statistically highly significant ($P<0.001$), indicating that the decrease in the adrenaline content of the adrenal gland producible by insulin hypoglycemia cannot be prevented by the 3rd–7th dorsal spinal roots section.

(3)

Eight toads were experimented on. On the right side, the splanchnic nerves were cut. On the left side, the 3rd–7th ventral spinal roots were severed. After 6 days the application of insulin was performed.

The adrenaline content per kg. of body weight in the left gland was estimated as 0.179 (0.138–0.270) mg. and that in the left gland was 0.180 (0.145–0.274) mg. The noradrenaline content per kg. was 0.096 (0.073–0.126) mg. in the left gland and 0.094 (0.061–0.133) mg. in the right gland. Difference between adrenaline estimates in the left and the right glands was statistically not significant ($P>0.20$). The depletion of adrenaline content in the adrenal gland causable by insulin hypoglycemia was able to be prevented by the previous section of the 3rd–7th ventral

spinal roots.

(4)

Five groups of animals were used. The splanchnic nerves were cut on the right side. Four ventral spinal roots out of five (the 3rd–7th) were severed on the left side, only one root being remained intact. Insulin was given 6 days after nerve sections.

Group A. In 8 toads the 3rd ventral spinal root was remained intact and the other 4 roots were cut. The adrenaline content of the left adrenal gland per kg. of body weight was estimated as 0.138 (0.062–0.186) mg. and that of the right gland was 0.204 (0.130–0.260) mg. The difference of estimates was statistically highly significant ($P<0.001$). The noradrenaline content per kg. was 0.141 (0.110–0.181) mg. and 0.146 (0.100–0.193) mg. in the left and right gland, respectively.

Group B. In 7 toads the 3rd, the 5th, the 6th, and the 7th ventral spinal roots were cut, leaving the 4th root intact. The adrenaline content per kg. was 0.127 (0.068–0.170) mg. in the left gland and 0.227 (0.176–0.270) mg. in the right gland. The difference was highly significant ($P<0.001$). The noradrenaline content per kg. was 0.146 (0.106–0.178) mg. and 0.149 (0.112–0.185) mg. in the left and the right gland, respectively.

Group C. Six toads were experimented on. The 5th ventral spinal root was kept intact and the other 4 roots were severed. The adrenaline content per kg. was 0.127 (0.069–0.197) mg. in the left adrenal gland and 0.208 (0.150–0.268) mg. in the right gland, respectively. The difference was statistically highly significant ($P<0.005$). The noradrenaline content per kg. of body weight was 0.124 (0.102–0.142) mg. and 0.133 (0.102–0.155) mg. in the left and the right gland, respectively.

Group D. Experiments were performed in 8 toads. The 3rd, the 4th, the 5th and the 7th ventral spinal roots were sectioned, leaving the 6th root intact. The adrenaline content per kg. in the left adrenal gland was 0.205 (0.158–0.305) mg. and that in the right gland was 0.207 (0.164–0.285) mg. There was no significant difference between the estimates ($P>0.50$). The noradrenaline content was 0.131 (0.089–0.149) mg. and 0.128 (0.095–0.144) mg. in the left and the right adrenal gland, respectively.

Group E. Eight toads were employed. The 3rd-6th ventral spinal roots were cut and the 7th root was kept intact. The adrenaline content per kg. of body weight was estimated as 0.180 (0.145–0.246) mg. in the left gland and as 0.183 (0.146–0.239) mg. in the right gland. There was no significant difference between the estimates ($P>0.50$). The noradrenaline content per kg. was 0.104 (0.068–0.129) mg. an 0.101 (0.077–

0.130) mg. in the left and the right adrenal gland, respectively.

By these experiments it was proved that the depletion in the adrenaline content of the adrenal gland causable by insulin hypoglycemia was not be prevented by the ventral spinal roots sections, if the 3rd, the 4th or the 5th root was remained intact. In contrast, the integrity of the 6th or the 7th ventral spinal root was found to have no effect on the prevention by the ventral root sections of the adrenaline depletion.

(5)

In 9 toads the 3rd, the 4th and the 5th ventral spinal roots were cut on the left side and the splanchnic nerves were sectioned on the right side. Six days after nerve sections the injection of insulin was made.

The adrenaline content, computed per kg. of body weight, was 0.165 (0.141–0.187) mg. and 0.167 (0.143–0.193) mg. in the right and the left adrenal gland, respectively. No significant difference was found between the estimates ($P>0.50$). The noradrenaline content per kg. was 0.095 (0.059–0.129) mg. and 0.098 (0.066–0.126) mg., respectively.

The results showed that the depletion of the adrenaline content of the adrenal gland producible by insulin hypoglycemia could be fully prevented by the sections of the 3rd–5th ventral spinal roots.

Discussion

In my previous work it was demonstrated that the adrenaline content of the adrenal gland in toads was decreased by insulin hypoglycemia and the section of the splanchnic nerves was capable of preventing the depletion of adrenaline content. These results indicated that the adrenaline-secretory fibers to the adrenal gland ran through the splanchnic nerves.

In the present investigation it was found at first that the decrease in the adrenaline content of the adrenal gland producible by insulin hypoglycemia was fully abolished by the 3rd–7th ventral and dorsal spinal roots section. The adrenaline-secretory fibers were thus proved to leave the spinal cord and run in some of these spinal roots. In the next step, the 3rd–7th ventral spinal roots section was made in one group of animals and the 3rd–7th dorsal spinal roots section in the other. The former was found to prevent the adrenaline depletion causable by insulin hypoglycemia, whereas the latter to have no effects in preventing the adrenaline depletion. Thus the adrenaline-secretory fibers were proved to run through some of these ventral spinal roots.

Then, further experiments were performed to decide the ventral spinal roots through which the adrenaline-secretory fibers run. When the 3rd ventral spinal root only was kept intact and the other 4 ventral roots, *i.e.* the 4th, the 5th, the 6th and the 7th, were cut, the depletion

of adrenaline content due to insulin hypoglycemia was not prevented by such nerve sections. The same was true, when the 4th or the 5th ventral spinal root only was left intact. On the other hand, when the 6th or the 7th ventral spinal root was kept intact and the other 4 roots were cut, the decrease in the adrenaline content producible by insulin hypoglycemia was entirely nullified. The section of the 3rd, the 4th and the 5th ventral spinal roots was found to be enough to prevent the adrenaline depletion in the adrenal gland causable by insulin hypoglycemia. Thus it is justifiable to conclude that the adrenaline-secretory fibers in toads run through the 3rd, the 4th and 5th ventral spinal roots.

As to the adrenaline-secretory fibers to the adrenal gland in dogs, Tournade and his co-workers[3] demonstrated that an increase in the adrenaline secretion was produced by the stimulation of D_4-L_1 ventral spinal roots but not by the centrifugal dorsal spinal root stimulation, indicating that the adrenaline-secretory fibers run through the ventral spinal roots. In cats Young[4] found histologically that the fibers to the adrenal medulla ran in D_6-D_{13} (L_3) ventral spinal roots. In the experiments of Wada *et al.*[5] on unanesthetized dogs, the augmented adrenaline secretion causable by morphine was found to be fully abolished by the section of ventral spinal roots from D_4 or D_5 to L_3 or L_4 but not by the dorsal root section.

From the experimental results of above investigators and those of the present investigation it was elucidated that in toads as well as in mammals the adrenaline-secretory fibers to the adrenal gland ran through the ventral spinal roots.

Summary

In toads the spinal root pathway of the adrenaline-secretory fibers to the adrenal gland was studied, taking the depletion of the adrenal gland as an indicator of augmented adrenaline secretion. The adrenaline and noradrenaline content of the adrenal gland was estimated by the use of the permaganate method.

The adrenaline-secretory fibers were found to run through the 3rd, the 4th and the 5th ventral spinal roots.

I wish to thank Prof. T. Suzuki for his help and encouragement, and for reading the manuscript.

References

1) Inoue, M., Tohoku J. Exp. Med., 1958, **68**, 279.
2) Suzuki, T. & Ozaki, T., *ibid.*, 1951, **54**, 332 ; Ozaki, T., *ibid.*, 1954, **61**, 83.
3) Tournade, A., Chabrol, M. & Wagner, P. E., Compt. Rend. Soc. Biol., 1925, **93**, 933.
4) Young, J. Z., J. Anat., 1939, **73**, 540.
5) Cited in Satake, Y., Tohoku J. Exp. Med., 1954, **60**, Suppl. II.

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Itinsulfuric Acid in Pig Skin

Second Report

Two Disaccharides Isolated from Skin Itinsulfate*

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Aizawa[1] claimed last year that skin itinsulfate, which Meyer and Rapport[2] named "chondroitinsulfate B", is a mixture of cartilage chondroitinsulfate and "dermoitinsulfate" which contains galacturonic acid as the uronic acid component. A spot was shown at the position corresponding to galacturonic acid, when he paper-chromatographed a HCl-hydrolysate of the skin itinsulfate of pig. But, that spot was probably L-iduronic acid which had been disintegrated by the hydrochloric acid remaining unexpelled by evaporation after the hydrolysis, because the writer found later a spot at a different position in a chromatogram of a sulfuric acid-hydrolysate of the skin itinsulfate, from which the sulfuric acid had been eliminated with barium carbonate and baryta, and as a matter of fact, Meyer and co-workers[3] identified it more recently with L-iduronic acid paper-chromatographically.

Moreover, whether skin itinsulfate is a mixture of chondroitin- and dermoitin-sulfate or mixtures of itinsulfates containing glucuronic and L-iduronic acids in various proportions is a problem unsolved despite of the attempts of Aizawa[1] and Meyer and others.[4] In 1957, Dorfman and others[5] pronounced the identity of skin itinsulfate with β-heparin. However, their skin itinsulfate (rabbit?) might have contained glucuronic acid besides L-iduronic acid as a hexuronic acid, and on the other hand, β-heparin from lung has never before been ascertained if it is free from glucuronic acid.

Apart from the question regarding the chemical entity of skin itinsulfate and β-heparin, Jeanloz *et al.*[6] methylated and methanolyzed β-heparin to isolate methyl 2-acetamido-2-deoxy-6-O-methyl-α-D-galactopyranoside,

* 222nd report of Masamune and co-workers' "Biochemical Studies on Carbohydrates. Read before the XXXIst Meeting of the Japanese Biochemical Society, Sapporo, July 14–16, 1958.

and suggested that, in β-heparin, 3- or 4-position of galactosamine is bound with hexuronic acid or sulfuric acid. And this year, Jeanloz and Stoffyn[7] stated that L-iduronic acid is attached to the 3-position, and sulfuric acid to the 4-position, because methyl 2-acetamido-2-deoxy-4,6-di-O-methyl-α-D-galactopyranoside is obtainable from fully methylated desulfated β-heparin. Meyer and others[8] digested so-called chondroitinsulfate B with β-heparin-adapted flavobacterium enzyme which had been heated at 56°C, and obtained Δ4–5 unsaturated di- and oligosaccharides, as when they treated cartilage chondroitinsulfate with the unadapted enzyme. Therefrom they claimed that chondroitinsulfate B contains the same β-4-hexosaminidic and β-3-uronidic linkages that are found in cartilage chondroitinsulfate. Dorfman and others[5] found that paper-chromatography of a HCl-hydrolysate of β-heparin, which they assumed to be identical with chondroitinsulfate B of Meyer, gave two galactosamine-containing spots correlated with 3-substituted hexosamine structure because of 510 mμ absorption maximum in the Elson-Morgan reaction.

The present account deals with two crystalline disaccharides separated from a fraction of skin itinsulfate. The material was prepared by successive fractional precipitation of skin itinsulfate of pig with ethanol in the presence of barium acetate, with a methanol-ethanol mixture in the presence of lead acetate and with methanol in the presence of zinc chloride in order to separate a fraction containing glucuronic acid as little as possible.

One of the disaccharides in question was identified with chondrosine by paper chromatography of its ninhydrin oxidation product as such and after hydrolysis and for the same optical rotation and infrared spectrum of it as those of chondrosine from chondroitinsulfuric acid of cartilage. The other disaccharide gave spots of L-iduronic acid and its lactone and galactosamine, when its hydrolysate was paper-chromatographed after fractioning by the aid of a cation-exchange resin. Its ninhydrin oxidation product showed spots at the positions corresponding to lyxose, L-iduronic acid and L-iduronolactone when hydrolyzed and paper-chromatographed, and gave the negative reaction with alkaline triphenyltetrazolium. Namely, the original disaccharide is plausibly assumed as galactosamine-3-L-iduronide. The infrared spectrum and the fact that it gave a neutral solution in water negated the presence of γ-lactone ring. It is named " dermosine " tentatively.

Experiments and Comments

Preparation of the Material

First stage. 2.64 kg. of fresh pig skin, shaved, mechanically defatted and minced, was digested with 6 *l.* of 1 *N* NaOH at 37°C with occasional shaking for 24 hours, neutralized with glacial acetic acid and filtered through Celite. The

filtrate (8.5 *l.*) was precipitated with 5.75 *l.* of ethanol after addition of 20 g. of crystalline barium acetate. The precipitate was centrifuged off, washed with ethanol and ether and dried in vacuo over $CaCl_2$. 25.7 g. of a grayish powder was given.

Second stage. It was kneaded with 150 cc. of 5% Na_2CO_3 in a homogenizer and centrifuged. The centrifugate was again homogenized with 50 cc. of the sodium carbonate solution and the centrifugal part here was further treated in a similar manner again and again. The total homogenates (300 cc.) were mixed with 150 cc. of 90% phenol in the homogenizer and centrifuged to give three layers. The upper watery layer which was strongly Goldschmiedt positive was separated. The middle and lower layers were washed with 5% Na_2CO_3, and the washing and the upper layer set aside were treated with phenol as above, employing 150 cc. of 90% phenol. The opalescent centrifuged upper layer here (350 cc.) was adjusted to pH 6.8 with 50% acetic acid, 2 volumes of abs. ethanol was added, and after 36 hours at room temperature, the mixture was centrifuged. The centrifugate was washed with ethanol and ether and dried. 2.4 g. of a white powder was thus yielded. 3.9 g. of a corresponding powder was obtained from another 2.5 kg. batch of pig skin. The two powders were united (6.3 g.) and dissolved in 200 cc. of 10% calcium chloride. The yellowish slightly turbid solution was adjusted to pH 7.8 with NaOH, deproteinized about 20 times by stirring with 150 cc. of an amylalcohol-chloroform (1:4 by volume) mixture in the homogenizer, until no gel-formation took place anymore, and 2 volumes of 95.5% ethanol was added under agitation. The deposit was stood overnight, centrifuged, washed with ethanol and ether and dried. 3.0 g. of a white powder was given.

This product was subjected to fractionations below to get a fraction containing the smallest possible amount of glucuronic acid, taking as a rough indication the color depth of the glucuronic acid spots after paper chromatography of the H_2SO_4-hydrolysates of fractions.

Third stage. It was dissolved in 100 cc. of water, passed through a 1.4×26 cm. column of Dowex 50 (hydrogen form) and washed with water. The effluent and washing (180 cc. in total) were received in a vessel containing 3 g. of crystalline barium acetate, the saturated baryta was added to pH 9.0, and after placing in a refrigerator (0--5°C) overnight, the mixture was centrifuged to discard some deposit that had occurred. The clear supernatant was neutralized with 1 *N* acetic acid and made up to 200 cc. with water, and abs. ethanol was added in drops under cooling on ice. The mixture containing 20% ethanol was stood in the refrigerator overnight and centrifuged. The centrifugate was washed with ethanol and ether and dried. A white powder given amounted to 2.9 g. This was changed into sodium salt by extraction with a sodium carbonate solution, into free acid by the aid of Dowex 50 and again into barium salt in turn. The solution here (220 cc.) was fractionally precipitated with ethanol three times as above, the precipitate at the ethanol concentration 0–10% being recovered each time, and turned into sodium salt by transferring its solution to a vessel containing sodium acetate through a Dowex 50 column resembling the one above. The effluent was precipitated with 2 volumes of ethanol. The

yield was 2.0 g.

Fourth stage. The product above was dissolved in 170 cc. of water and adjusted to pH 4.9 with dilute acetic acid, and 30 cc. of 10% lead acetate was added. The mixture was stood for 1 day in the refrigerator. Slight turbidity occurred but could not be removed by centrifugation, so that the clowdy solution was immediately precipitated by addition of a mixture of methanol and ethanol (2:1 by volume) to 20% under cooling. After standing overnight at 0°C, the gelatinous deposit was centrifuged, washed with 10% lead acetate, to which the organic mixture above had been added to 20%, ethanol and ether and dried. 2.8 g. of a powder was given. It was changed into sodium salt as above. Yielded was 1.9 g. of a white powder.

Fifth stage. The fractionation procedure which Marbet and Winterstein[9] devised for preparing β-heparin was applied as follows. The substance obtained in the fourth stage was dissolved in 110 cc. of 10% zinc chloride, methanol was added to 45% at room temperature, and the mixture was centrifuged after 3 hours. The centrifugate was washed with methanol and ether and dried. The powder given (1.77 g.) was reprecipitated as above, and the powder here (1.22 g.) was taken up in 100 cc. of water, passed through a 1.4×6.5 cm. column of Dowex 50 (hydrogen form) and washed with water. To the effluent and washing was added sodium acetate (crystalline) to 1%. This mixture was adjusted to pH 7.2 with dilute NaOH and made up to 150 cc. with water, and 300 cc. of ethanol was added. The deposit was centrifuged after standing overnight in the refrigerator, washed with ethanol repeatedly and with ether and dried to a constant weight in vacuo over anhydrous calcium chloride. 1.15 g. was the sodium salt (a white powder) given.

Even after these successive fractionations the product was still found to contain a small amount of glucuronic acid. This was used as the material for isolation of the disaccharides.

Properties of the Final Product

It showed a single boundary after electrophoresis in either of acetate buffer of pH 4.6 and I 0.2 and phosphate buffer of pH 7.7 and I 0.2, and also proved ultracentrifugally homogeneous in those buffers (In the case of the phosphate buffer, I was raised to 2.0 with NaCl). The paper chromatograms of its hydrolysate showed distinct spots at the positions corresponding to galactosamine and L-iduronic acid and a faint spot at the position corresponding to glucuronic acid. The analytical figures obtained are embodied in Table I. $[\alpha]_D^s$ $(H_2O)=\dfrac{-0.699\times100}{1.08\times1}=-64.8°$ (c 1.08).

Isolation of Chondrosine and Dermosine

1 g. of the material (sodium salt) was dissolved in 100 cc. of water, passed through a 1.4×6.5 cm. column of Dowex 50 (hydrogen form) and washed with water. To the effluent and washing united were added 2 g. of crystalline barium acetate and 1/4 the volume of ethanol in turn, followed by standing overnight. The centrifugate was washed with ethanol and ether and dried in vacuo over

TABLE I

Composition of the Material

Analysis	In per cent	In equivalents per equivalent weight
N*	2.60	1.08
Galactosamine†	30.5	1.00
Hexuronic acid as L-iduronic acid		
Orcinol‡	33.8	1.03
Indole§	33.7	1.02
Acetyl//	6.97	0.94
Hydrolyzable sulfur¶	5.5	1.00
Ash (Na_2SO_4)**	22.8	0.94

* Micro Kjeldahl. † Masamune and Yosizawa[10]. ‡ A modification of Tillmans, Philippi and Ozaki method[11]. § Dische[12]. ‡, § The standard solution was prepared by heating 1,2-O-isopropylidene-L-iduronolactone, which had been kindly given from Prof. M. L. Wolfrom, with 0.1 *N* H_2SO_4 at 62°C for 10 minutes. // Suzuki[13]. ¶ Suzuki[13]. ** Pregl.

$CaCl_2$. The barium salt (1.1 g.) thus given was dissolved in 50 cc. of 0.5 *N* H_2SO_4, sealed in a tube and heated at 100°C for 6 hours[14]. When cold, the hydrolysate was freed from sulfuric acid by adding 2.2 g. of barium carbonate in portions at first and afterwards adding the saturated baryta until no more cloudiness occurred. The deposit was centrifuged and washed with water at 60–70°C repeatedly. The yellowish clear supernatant (pH 4.2) and washing (200 cc. in total) were fractionated by column chromatography, chiefly adapting the method for isolating chondrosine from chondroitinsulfate of Davidson and Meyer.[15] Namely, the fluid mixture was added to a 1.4×12 cm. column of Dowex 50 (hydrogen form, mesh>150) and eluted with 200 cc. of water at first and then with 0.005 *N* acetic acid at a flow-rate of 1–1.5 cc. per minute (Room temperature 2–5°C). The effluent was collected in 16 cc. fractions, and an 0.2 cc. portion of the fractions were diluted to 1 cc. and tested with 3 cc. of the orcinol reagent of Brown[16], giving the curve plotted in Fig. 1. The contents of tubes Nos. 40–70 showing the first peak and those of tubes Nos. 75–175 showing the second peak were united separately (Div. I and II). The both divisions were distilled in vacuo at 30°C. From Div. I appeared crystals in needles during the distillation. The crystalline porridge given was stood for a while and filtered. The light brown crystals separated amounted to 34 mg.—Disaccharide I. Div. II gave no crystals during the distillation. Therefore, the yellowish brown syrup was dissolved in 20 cc. of water and again fractionated by column-chromatography in a similar manner, having shortened the length of column to 6.5 cm. and employing 100 cc. instead of 200 cc. of water for preliminary washing. The 0.005 *N* acetic acid eluate was collected in 10 cc. fractions, and Brown orcinol method was applied in the manner above. The fractions showed a reaction peak in Fig. 2.

The contents in tubes Nos. 60–120 were condensed together to about 200

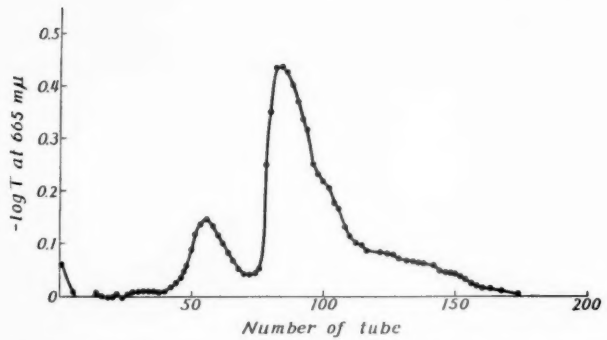

Fig. 1. Elution pattern of the hydrolysate of the skin itinsulfate as expressed in optical density after Brown reaction of the fractions.

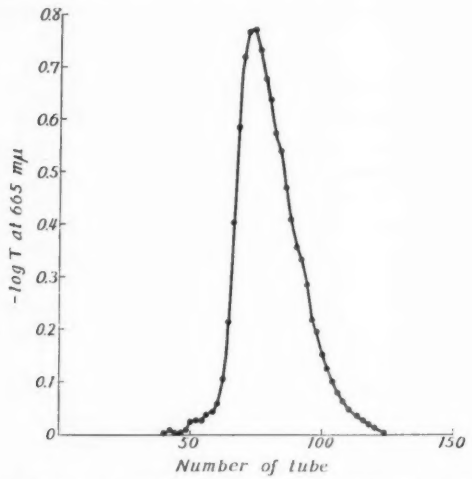

Fig. 2. Elution pattern of Div. II rechromatographed as expressed in optical density after Brown reaction of the fractions.

cc. and lyophilyzed. 60 mg. of a white powder was given. It was dissolved in 0.1 cc. of water and stood, whereupon crystallization took place in a few minutes. The mixture containing crystals was cooled on ice, 1.5 cc. of 50% ethanol was added, and the crystals were centrifuged and washed with 2 cc. of chilled 50% ethanol and 1 cc. each of abs. ethanol and acetone. 30 mg. of crystals in rod (or whetstone) form was given. The product began to brown at 160°C and decomposed at about 166°C. It was recrystallized from 0.35 cc. of water.—Disaccharide II. Yield 22 mg. F.P. 170° (decomp.). From the mother fluid of the crude crystals above was obtained 29 mg. of crystals in rods melting at

166°C under decomposition. This portion was also recrystallized. See the crystalline form in Fig. 3.

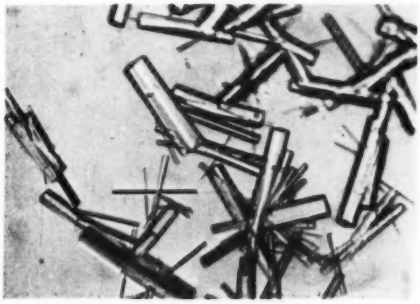

Fig. 3. Microphotograph of Disaccharide II (dermosine).

Experiments with the Disaccharides

Disaccharide I. *1) Solubility.* It dissolved slightly in cold water but fairly in hot water. It dissolved scarcely in ethanol.

2) Test-tube tests. Molisch −, Goldschmiedt +.

3) Paper partition chromatography. Its paper chromatograms were developed on Toyo Roshi filter paper No. 3 by descending irrigation with either of the solvents below and indicated with aniline phthalate reagent of Partridge. It gave a spot with $R_{glucose}$ 0.09 or $R_{lactose}$ 0.18 when solvent butanol-pyridine-water (5:3:2)[17] was used, and a spot with $R_{glucose}$ 0.25 or $R_{lactose}$ 0.87 when solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1)[17] was used.

4) Ninhydrin oxidation. The disaccharide was oxidized with ninhydrin under the conditions preferred by Stoffyn and Jeanloz[18] excepting that duration of heating was extended to 1 hour:— 2 mg. of it was dissolved in 0.1 cc. of 2% ninhydrin in water containing 4% pyridine, sealed and heated at 100° for 1 hour. When cold, the solution was diluted with water to 1 cc. and washed with three 1 cc. portions of chloroform, three 1 cc. portions of butanol and five 1 cc. portions of ether in succession, and the ether remaining in the solution was expelled by evacuation. Next it was passed through a 0.7×1.3 cm. column of Dowex 50 (hydrogen form) and washed with water thoroughly. The effluent and washing (15 cc. in total) were distilled in vacuo at 30°C nearly to dryness, dissolved in 0.08 cc. of water and chromatographed on a sliver of Toyo Roshi filter paper No. 3, having sampled two 0.02 cc. portions of the solution side by side. Solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1).[17] Irrigation descending; temperature 20–21°C; duration of test 11 hours. The chromatogram was dried and scissored longitudinally, and one of the halves was sprayed with aniline phthalate reagent of Partridge (*a*) and the other with the triphenyltetrazolium reagent of Wallenfels[19] (*b*). In the case of indicator *a* a marked reddish brown spot with $R_{glucose}$ 0.51 ($R_{lactose}$ 1.55; $R_{maltose}$ 1.33) and a faint spot at the position corresponding to glucuronic

acid were shown but no spot at the position corresponding to lyxose. In the case of indicator *b* a faint red spot only was noticed at the position corresponding to glucuronic acid but no spot else. See Fig. 4.

Therefore, the remaining half of the solution, a half of which had been chromatographed above, was distilled in vacuo at 30°C. The syrupy residue was taken up in 0.2 cc. of 1 *N* HCl and heated for 13 hours in a sealed tube at 100°C. The hydrolysate was liberated from the hydrochloric acid by evaporating in vacuo over solid caustic soda at 35°C, adding water from time to time, and finally dissolved in 0.04 cc. of water. And a half of the solution was put on a sliver of paper (Toyo Roshi filter paper No. 3) to develop an *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1)[7] chromatogram by 9 hours' descending irrigation at 20–21°C. Lyxose, glucuronic acid and glucuronolactone were indicated when sprayed with Partridge aniline phthalate reagent (Fig. 5), that is, the galactosamine residue was proved to stand at the reducing end of Disaccharide I. In conjunction with the former result that the reagent of Wallenfels did not react with the unhydrolyzed oxidation product contrary to that of Partridge reagent, the present result strongly suggests a structure of galactosamine-3-glucuronide.

Fig. 4

Fig. 5

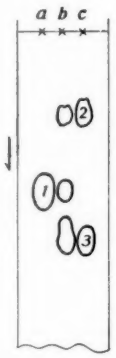

Fig. 4. Paper chromatograms of Disaccharide I treated with ninhydrin. Reference runs were carried out with 1% solutions of lactose, of lyxose and of glucuronic acid, which had been placed for 1 day in a refrigerator after preparation. (**I**) Indicated with the aniline hydrogen phthalate reagent of Partridge. (**II**) Indicated with the triphenyltetrazolium reagent of Wallenfels.

a, *d*: reference. *b*, *c*: Disaccharide I after ninhydrin oxidation. *1* lactose, *2* glucuronic acid, *3* lyxose, *4* glucuronolactone.

Fig. 5. Paper chromatogram of the hydrolysate of Disaccharide I which had been oxidized with ninhydrin. For reference were used 1% solutions of lyxose and of glucuronic acid, which had been stood for 1 day at 0°C after preparation. Indicator aniline hydrogen phthalate of Partridge.

a, *c*: reference, *b*: the hydrolysate. *1* lyxose, *2* glucuronic acid, *3* glucuronolactone.

5) It agreed with chondrosine from cartilage chondrotinsulfuric acid ($[\alpha]_D^{19}=\frac{+0.355\times100}{0.88\times1}=+40.3°$) in optical rotation in water.

$$[\alpha]_D^{25}=\frac{+0.249\times100}{0.62\times1}=+40.5° \text{ (no mutarotation)}$$

6) *Infrared spectrum* (The writer is indebted to Mr. S. Aono in the Chemical Institute of Non-aqueous Solutions). The infrared spectrum was recorded on a Perkin-Elmer Model 21 infrared spectrophotometer with a NaCl prism using the KBr disk method. The spectrum coincides with that of chondrosine prepared from chondroitinsulfuric acid of cartilage according to Davidson and Meyer.[15] See Fig. 6.

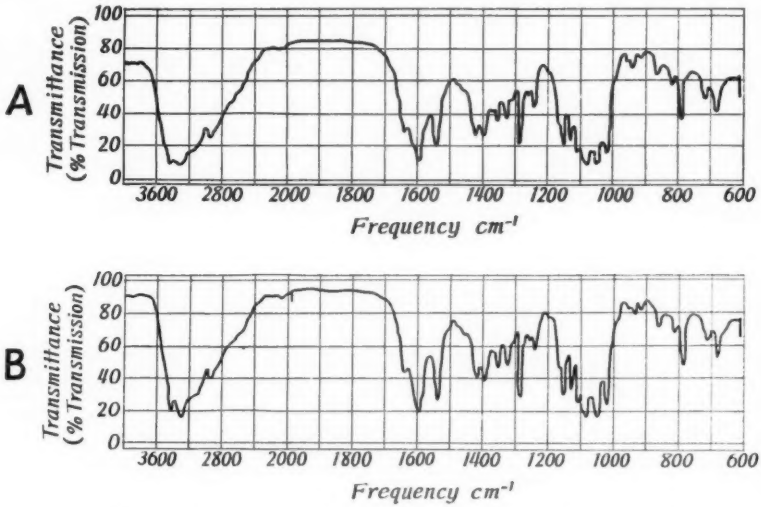

Fig. 6. Infrared spectra of chondrosine from cartilage chondroitinsulfuric acid (**A**) and of Disaccharide I (**B**).

In short, the findings *1*)–*6*) all point to the identity of Disaccharide I with chondrosine.

Disaccharide II. *1*) *Solubility*. It dissolved in water more easily than Disaccharide I, but did not dissolve in ethanol.

2) *Test-tube tests*. Molisch –, Goldschmiedt +.

3) *Paper chromatography*. Paper partition chromatography was processed as with Disaccharide I, and a spot with $R_{glucose}$ 0.12 or $R_{lactose}$ 0.25 was given when irrigated with solvent butanol-pyridine-water (5:3:2)[17] and a spot with $R_{glucose}$ 0.41 or $R_{lactose}$ 1.37 when irrigated with solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1)[17].

4) *Paper chromatography after acid hydrolysis*. 2 mg. of substance was heated

for 14 hours with 0.5 cc. of 1 *N* H_2SO_4 at 100°C in a sealed tube. The saturated baryta was added to the hydrolysate to pH 6.4, the deposit was centrifuged off and washed repeatedly with water at 60–70°C, and the supernatant and washings were united, passed through a 0.7×2.6 cm. column of Dowex 50 (hydrogen form) and washed. The effluent and washing (20 cc. in total) were distilled in vacuo at below 30°C to dryness and dissolved in 0.04 cc. of water to develop a paper-cnromatogram of uronic acid with 0.02 cc. of the solution (Fig. 7). Paper sliver Toyo Roshi filter paper No. 3. Solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1)[17]. Irrigation descending. Temperature 20–22°C. Duration

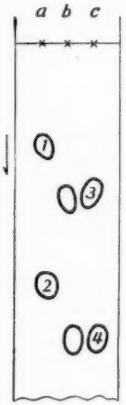

Fig. 7. Uronic acid paper chromatogram of Disaccharide II. Reference runs were carried out with 1% solutions of hexuronic acids, which had been placed for 1 day in a refrigerator after preparation. Indicator *p*-anisidine phosphate reagent of Mukherjee and Srivastava.[20]

a, *c*: reference. *b*: hexuronic acid fraction of Disaccharide II. *1* glucuronic acid, *2* glucuronolactone, *3* L-iduronic acid, *4* L-iduronolactone.

of test: 9 hours. Indicator *p*-anisidine phosphate (Mukherjee and Srivastava[20]). Two spots were shown at the positions corresponding to L-iduronic acid[21] (strong) and its lactone (weak). Next, the column on which the hexosamine had been adsorbed was washed with 20 cc. of 1 *N* HCl, and the eluate was distilled under reduced pressure at below 40°C. The dry residue did not smell of hydrochloric acid. A half of it was put on a sliver of Toyo Roshi filter paper No. 3 and chromatographed by the analytical method of Masamune and Yosizawa[22]. Temperature 24°C. Duration of test 24 hours. Spray reagent aniline phthalate. A spot was shown at the position corresponding to galactosamine hydrochloride. From these findings, the disaccharide is assumed to be composed of galactosamine and L-iduronic acid.

5) *Quantitative analysis.* The analytical figures obtained are embodied in Table II. It is shown that L-iduronic acid is colored by the indole reaction as 1.1 times intensively as by the orcinol reaction as expressed as glucuronic acid.

6) *Ninhydrin oxidation.* It was oxidized with ninhydrin and paper-chromatographed without and with previous hydrolysis, as Disaccharide I was. In the first case Partridge reagent indicated a marked reddish brown spot with $R_{glucose}$ 0.76 ($R_{lactose}$ 2.52; $R_{maltose}$ 2.42) and a faint spot at the position corresponding to L-iduronic acid but no spot at the position corresponding to lyxose, while Wallenfels reagent showed only a faint red spot at the position

TABLE II

Composition of Disaccharide II

Analysis	Found (%)	Calcd. for $C_{12}H_{21}O_{11}N \cdot 2H_2O$ (%)
Crystal water	5.32*	9.23
N†	3.61	3.58
C‡	36.84	36.83
H‡	6.50	6.44
Galactosamine§	41.0	45.6
Uronic acid as L-iduronic acid Orcinol//	45.7	49.8
Indole¶	44.5	
Iodine use as glucose**	103.9††	

* 7.591 mg. of the disaccharide was dehydrated at 105°C over P_2O_5 for 3 hours to constant weight 7.187 mg., that is, the decrease (0.404 mg.) disagreed with the calculated value (0.701 mg.) from the C and H contents. † Micro Dumas. ‡ Pregl. †, ‡ The writer is indebted to Mr. H. Obara in the Chemical Department. § Masamune and Yosizawa[10]. // A modification of Tillmans, Philippi and Ozaki method[11]. ¶ Dische[12]. ** Macleod and Robison[23]. †† The corresponding value of chondrosine 104.4.

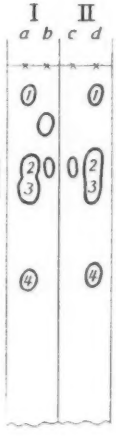

Fig. 8. Paper chromatograms of Disaccharide II (dermosine) treated with ninhydrin. Reference runs were carried out with 1% solutions of lactose, of lyxose and of L-iduronic acid, which had been placed for 1 day in a refrigerator after preparation. (**I**) Indicated with the aniline hydrogen phthalate reagent of Partridge. (**II**) Indicated with the triphenyltetrazolium reagent of Wallenfels.

a, *d*: reference. *b*, *c*: Disaccharide II after ninhydrin oxidation. *1* lactose, *2* L-iduronic acid, *3* lyxose, *4* L-iduronolactone.

corresponding to L-iduronic acid. See Fig. 8. In the case of the hydrolysate, lyxose, L-iduronic acid and L-iduronolactone were indicated by Partridge reagent (Fig. 9), revealing that the galactosamine residue stands at the reducing end of Disaccharide II.

Namely, Disaccharide II is regarded to have a structure of galactosamine 3-L-iduronide.

7) *Optical rotation in water.*

Fig. 9. Paper chromatogram of the hydrolysate of Disaccharide II (dermosine) which had been oxidized with ninhydrin. For reference were used 1% solutions of arabinose, of lyxose, of glucuronic acid and of L-iduronic acid, which had been stood for 1 day at 0°C after preparation. Indicator aniline hydrogen phthalate reagent of Partridge.

a, *c*: reference. *b*: the hydrolysate. *1* arabinose, *2* lyxose, *3* glucuronic acid, *4* L-iduronic acid, *5* glucuronolactone, *6* L-iduronolactone.

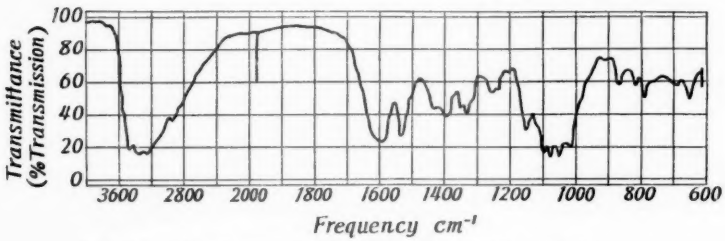

Fig. 10. Infrared sepctrum of Disaccharide II (dermosine).

$$[\alpha]_D^{13} = \frac{+0.306 \times 100}{0.76 \times 1} = +40.3°$$

$$[\alpha]_D^{17.5} = \frac{+0.453 \times 100}{1.16 \times 1} = +39.0°$$

No mutarotation occurred.

8) *Infrared spectrum* (The writer is indebted to Mr. S. Aono in the Chemical Institute of Non-aqueous Solutions). The infrared absorption was examined like on Disaccharide I. See Fig. 10. Since no absorption occurred at 1800 cm.$^{-1}$–1700 cm.$^{-1}$, γ-lactone ring is excluded.

Summary

A fraction containing the least possible quantity of glucuronic acid was separated from skin itinsulfate of pig, and two disaccharides chondrosine and " dermosine " which is galactosamine-3-L-iduronide were isolated from its hydrolysate. The properties of those disaccharides are described.

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References and Notes

1) Aizawa, Tohoku J. Exp. Med., 1957, **65**, 375.
2) Meyer & Rapport, Science, 1956, **113**, 596.
3) Hoffman, Linker & Meyer, *ibid.*, 1956, **124**, 1252.
4) Hoffman, Linker & Meyer, Arch. Biochem. & Biophys., 1957, **69**, 435.
5) Cifonelli, Ludowieg & Dorfman, Fed. Proc., 1957, **16**, 165.
6) Jeanloz, Stoffyn & Tremege, *ibid.*, 1957, **16**, 201.
7) Jeanloz & Stoffyn, *ibid.*, 1958, **17**, 249.
8) Hoffman, Linker, Sampson, Meyer & Korn, Biochem. Biopys. Acta, 1957, **25**, 658.
9) Marbet & Winterstein, Helv. chim. Acta, 1951, **34**, 2311.
10) Masamune & Yosizawa, Tohoku J. Exp. Med., 1957, **65**, 169.
11) Ozaki, J. Biochem. (Japan), 1936, **24**, 73. The reaction mixture was so modified in composition as to consist of 10 cc. of the sulfuric acid reagent, 0.5 cc. of the orcinol reagent containing 1.8% orcinol and 1 cc. of the unknown solution, and the extinction at 424 mμ was measured after heating.
12) Dische, Mikrochemie, 1929, **7**, 33. The extinction at 462 mμ was measured instead of colorimetry.
13) Suzuki, J. Biochem. (Japan), 1938, **27**, 367.
14) Shorter heating is probably more advisable to prepare Disaccharide II (" dermosine "), because no small amount of L-iduronic acid which constitutes that disaccharide becomes free (paper chromatography) soon in the early stage of the acid hydrolysis.
15) Davidson & Meyer, J. Am. Chem. Soc., 1954, **76**, 5686.
16) Brown, Arch. Biochem., 1946, **11**, 269. A 1 cc. sample solution was heated with 3 cc. of the orcinol reagent in an acetylation tube of Masamune and Yosizawa[10]. Extinction was measured at 665 mμ.
17) Masamune & Yosizawa, Tohoku J. Exp. Med., 1953, **59**, 1.
18) Stoffyn & Jeanloz, Arch. Biochem. & Biophys., 1954, **52**, 373.
19) Wallenfels, Naturwissenschaften, 1950, **21**, 491.
20) Mukherjee & Srivastava, Nature, 1952, **169**, 330.
21) L-Iduronic acid used for reference was prepared from 1,2-O-isopropylidene-L-idurono-lactone.
22) Masamune & Yosizawa, Tohoku J. Exp. Med., 1956, **64**, 53.
23) Macleod & Robison, Biochem. J., 1929, **23**, 517.